is part completes Volume 78 (1961)
PRIL 1961, VOLUME 78, No. 4

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> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland)—(Abstract).

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Footnotes. These should be avoided in the text as far as possible.

CORRIGENDA

Barbiturates and oxidative phosphorylation

By W. N. Aldridge and V. H. Parker

Volume 76 (1960)

p. 47, col. 2, last line

for KCl (10 mM) *read* KCl (0·1 M)

p. 48, col. 1, line 1

for EDTA (mM) and sucrose (30 mM)

read EDTA (mM), glycylglycine (16·7 mM) and sucrose (30 mM). This solution was adjusted to pH 6·7–6·8 with KOH.

Ion-exchange reactions between cartilage and various cations

By J. R. Dunstone

Volume 77 (1960)

p. 165, Table 1, line 2

for (sulphate content, 0·81 m-equiv./l.)

read (sulphate content, 0·81 m-equiv./g.)

The incorporation of radioactive amino acids into proteins of the microsome fraction of guinea-pig liver at very short time intervals after administration

By J. L. Simkin and C. R. Sutton

Volume 77 (1960)

p. 490, col. 1, line 18

delete oven

Thiosulphate Oxidation and Cytochromes in *Thiobacillus X*

1. FRACTIONATION OF BACTERIAL EXTRACTS AND PROPERTIES OF CYTOCHROMES

BY P. A. TRUDINGER
Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia

(*Received* 23 *March* 1960)

In a preliminary communication (Trudinger, 1958) the separation of some soluble cytochromes of the autotrophic sulphur bacterium *Thiobacillus X* was briefly reported. Addition of thiosulphate to intact cells results in the rapid reduction of the cytochromes and the present work was undertaken to attempt to characterize the cytochromes and to obtain some information on the electron-transport chain involved in the oxidation of thiosulphate.

This paper describes in detail the separation and some properties of the soluble cytochromes.

MATERIALS AND METHODS

Organism and growth conditions. The organism used in this study was *Thiobacillus X*, isolated and described by Parker (1947) and Parker & Prisk (1953). The bacteria were grown at 26° in the medium described by Vishniac & Santer (1957) for the culture of *Thiobacillus thioparus*, with thiosulphate as an energy source. Sufficient bromocresol purple was added to the medium to give an observable colour, and saturated Na_2CO_3 was added from time to time to keep the pH between 6 and 8. Un-neutralized cultures of *Thiobacillus X* carried out a partial oxidation of thiosulphate and growth ceased when the pH of the medium reached approx. 3 (Parker & Prisk, 1953). With neutralization, however, growth continued until all substrate was converted into sulphate.

Small amounts of bacteria were grown in penicillin flasks with mechanical shaking. Large batches of bacteria were grown in carboys containing 15 l. of medium, through which was forced air containing about 5% of CO_2. After incubation at 26° for 3–4 days the bacteria were harvested by centrifuging and washed twice with 0·1 M-phosphate, pH 7. When not used immediately the cells were freeze-dried and stored at 2°.

Mammalian cytochrome c. This was prepared from ox heart and was kindly donated by Dr C. A. Appleby.

Haemochromogens. Pyridine and cyanide haemochromogens of cytochromes were prepared according to Vernon & Kamen (1954).

Spectroscopy and spectrophotometry. Visual examinations of absorption spectra were made with a Beck–Hartridge reversion spectroscope or a Beck model 3000 microspectroscope. The latter apparatus is fitted with a comparison prism. For low-temperature spectra materials were mixed with an equal volume of glycerol, frozen in liquid air in a layer 1–2 mm. thick, and examined in the Beck spectroscope after devitrification (Keilin & Hartree, 1949, 1950).

Spectral curves were obtained with a Beckman model DU spectrophotometer with 1 cm. cells.

Terminology. The cytochromes described in this paper have been named according to their source and the locations of their α-absorption peaks in the reduced state (Scarisbrick, 1947). Different components with similar spectra have been distinguished by means of a number in parentheses (cf. Gibson & Larsen, 1955).

Chromatography on Amberlite IRC-50. Commercial Amberlite IRC-50 (XE97) was purified by washing successively with 2 N-NaOH, water, 2 N-HCl and water. Small batches (20–30 g.) of the purified resin were then stirred for 1 hr. with 1 l. of either 0·02 M-potassium phosphate, pH 7, or 0·05 M-sodium citrate, pH 5, and filtered. This procedure was repeated (generally five or six times) until the pH of the buffer changed by less than 0·1 unit. The resin was packed to a depth of 10 cm. in a 1 cm.-diameter column equipped with a jacket through which ice–water was passed. Materials applied to the column were dissolved in the buffer with which the resin was washed and were eluted with increasing buffer concentrations at the rate of 0·3–0·5 ml./min. Protein and cytochrome elution were followed by measuring the extinctions at 280 and 410 mμ respectively of 5 ml. fractions.

Paper electrophoresis. Electrophoresis of cytochromes was carried out in an EEL apparatus (Evans Electroselenium Ltd., Harlow, Essex), with 42 cm. × 1 cm. strips of Whatman no. 1 paper and 0·05 M-potassium phosphate buffers. A voltage of 200 v was applied for 16 hr. Small amounts of cytochrome were detected by spraying the papers with saturated benzidine containing 1% (v/v) of H_2O_2.

Assay of thiosulphate-oxidizing enzyme. The assay is based on results described by Trudinger (1961). The material to be analysed was mixed in a 1 cm.-diameter tube with 0·12 M-potassium phthalate, pH 5, 5 μmoles of $K_3Fe(CN)_6$ and 40 μmoles of $Na_2S_2O_3$. The final volume was 5 ml. and the temperature 23°. E at 420 mμ was recorded at 0·5 min. intervals with a Unicam SP. 600 spectrophotometer. Active enzymic fractions were diluted if necessary until the rate of reduction of ferricyanide was less than 1 μmole/2 min. Under these conditions the reduction rate was approximately linear for 2–3 min. Activities were determined from the linear portion of the curve. One unit of enzyme is defined as the amount required to reduce 1 μmole of ferricyanide/min.

Oxidation–reduction potentials of cytochromes. These experiments were carried out at 30° with the Beckman spectrophotometer and 1 cm. light-path cuvettes fitted with Thunberg side bulbs. Sufficient cytochrome to give a final $E_{\alpha\max.}$ in the reduced state of 0·2–0·4 was mixed with 0·1–0·15 μmole of either 2:6-dichlorophenol-indophenol or *o*-chlorophenol-indophenol (E'_0, pH 7, 30° +0·217 v and +0·233 v respectively) in a final volume of 3 ml. of 0·1 M-phosphate, pH 7. The side bulb contained a small amount of ascorbate, sufficient to cause a partial reduction of cytochrome, and dye. This was tipped into the reaction mixture after evacuation of the system with a high-vacuum pump. Equilibrium was reached within 2–3 min. The concentrations of oxidized 2:6-dichlorophenol-indophenol and *o*-chlorophenol-indophenol were determined from the values of $E_{605\,m\mu}$ and $E_{620\,m\mu}$ respectively, after correction for the absorption due to oxidized cytochrome at these wavelengths. Although the absorption by cytochrome at 605 and 620 mμ decreased as the cytochrome was reduced the change was insufficient to cause a significant error in the estimation of dye. The relative concentrations of ferricytochrome and ferrocytochrome were calculated from the values of $E_{\alpha\max.}$, after correction for the absorption due to oxidized dye, and the standard potentials of the cytochromes were derived from the normal electrode equation.

Preparation and fractionation of bacterial extracts. All operations in this and subsequent fractionations were carried out at 0–5°. Except for differences in the enzymic activity of particles, similar results were obtained with fresh and freeze-dried bacteria.

The bacteria, 5–8 g. dry wt., were suspended evenly in 30 ml. of 0·04 M-potassium phosphate buffer, pH 7, and passed twice at 15 000 lb./in.^{2} through the apparatus described by Milner, Lawrence & French (1950). This procedure disrupted more than 95% of the cells. The mixture was made up to 100 ml. with buffer, stirred overnight at 2° and centrifuged at approx. 8000 *g* for 30 min. The precipitate, fraction P_1, was usually discarded and the supernatant centrifuged again at 40 000 rev./min. (145 000 *g* at the bottom of the tube) for 60 min. in the no. 40 head of a model L Spinco centrifuge. The supernatant was collected and the particles (fraction P_2) were resuspended in 50 ml. of buffer and centrifuged again under the same conditions.

EXPERIMENTAL AND RESULTS

Spectra of intact cells

Fig. 1 shows a difference spectrum between oxidized intact cells of *Thiobacillus X* and cells in the presence of $Na_2S_2O_3$. Thiosulphate caused an instantaneous reduction of a number of cytochrome components. At room temperature the reduced α-absorption region of Fig. 1 appeared in the Beck–Hartridge spectroscope as a broad intense band from 549 to 558 mμ with a faint band at 562 mμ. At the temperature of liquid air aerated cells showed absorption bands at 510–530 mμ and at 558–562 mμ. In the presence of $Na_2S_2O_3$ a number of absorption bands appeared, the major bands being listed in Table 1. A number of very faint bands were also observed in the regions of 510 and 530 mμ, but these were too indistinct to be defined accurately. No absorption bands above 560 mμ were detected.

All cytochrome absorption bands of thiosulphate-reduced cell suspensions were slightly intensified by the addition of $Na_2S_2O_4$ (e.g. the height of the α peak of the spectrum shown in Fig. 1 was increased by about 10%). No additional bands were observed in the presence of $Na_2S_2O_4$.

Separation of cytochromes and thiosulphate-oxidizing enzyme

Extracts of bacterial cells after high-speed centrifuging showed spectra qualitatively similar to those of intact cells with the exception that the band in the reduced state at 562 mμ was missing. The extracts were fractionated as shown in Fig. 2. At each stage fractions were examined with the hand spectroscope in the presence of $Na_2S_2O_4$ and tested for thiosulphate-oxidizing activity by the standard test described above.

Fractionation with ammonium sulphate. Although only a partial separation of cytochromes and thiosulphate-oxidizing enzyme was achieved by this treatment, subsequent fractionation on Amberlite IRC-50 was much facilitated. Fraction AS 40–70 contained all the recovered enzyme activity together with a mixture of cytochrome components. Fractions AS 70–95 was enriched with respect to those cytochromes absorbing between 552 and 558 mμ.

Chromatography on IRC-50 at pH 7. At pH 7, the thiosulphate-oxidizing activity of fraction AS 40–70 was adsorbed on to Amberlite IRC-50 from 0·02 M-

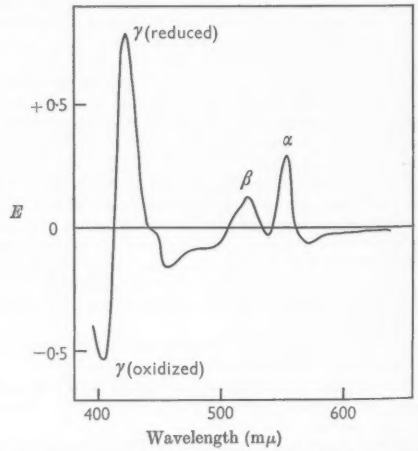

Fig. 1. Difference spectrum of intact *Thiobacillus X* (5 mg. dry wt./ml.) reduced with thiosulphate against oxidized cells. The positions of the absorption peaks are at α, 553 mμ; β, 522 mμ; γ (reduced), 422 mμ and γ (oxidized), 403 mμ.

Table 1. *Low-temperature spectra of intact cells and soluble cytochromes*

The reduced materials in 50% glycerol were examined with the hand spectroscope at liquid-air temperature. Wavelengths of the absorption bands (mμ) and their approximate relative intensities (in parentheses) are given.

Region	Intact cells	Cytochrome 550 (1)	Cytochrome 553·5	Cytochrome 557 (1)	Acidic fraction
α	547–549 (+ + + + +)	547–549 (+ + + + + −)	550–552 (+ + + + +)	553–556 (+ + + + +)	547–549 (+ + + + +)
	550–556 (+ + + + +)	—	—	—	—
	560 (+)	—	—	—	—
	—	537 (+)*	532 (+)*	532–533 (+)*	—
β	525 (+ +)	525 (+ + +)	522–523 (+ + +)	524 (+ + +)	524–525 (+ + +)
	519–521 (+ +)	519 (+ + +)	520 (+)	519 (+)	519 (+)
	—	508 (+)	510 (+)	510–511 (+)	—

* These bands were tentatively assigned to the α region by analogy with mammalian cytochrome *c* (Estabrook, 1956).

phosphate and eluted by 0·1 M-phosphate. The cytochromes (fraction AMB 1) passed through the resin column. Provided that only small amounts (5–10 mg. dry wt.) of fraction AS 40–70 were applied to the column, a complete separation of oxidizing enzyme from cytochromes was achieved at this stage. However, it was more rapid and convenient as a routine procedure to process larger amounts (up to 100 mg. dry wt.) of fraction AS 40–70. Under these conditions the fraction containing enzyme (AMB 2) was still contaminated with small amounts of cytochrome, which was removed by a second treatment with IRC-50. The yield of enzyme in this case was about 20% less than in the single-step separation.

Two cytochrome components of fraction AS 70–95 were adsorbed from 0·02 M-phosphate on IRC-50 at pH 7. These were eluted by 0·1 M- and 0·2 M-phosphate and have been named *Thiobacillus X* cytochromes 557 (1) and 550 (1) respectively. The unadsorbed material had similar spectroscopic properties to fraction AMB 1, with which it was combined. It was sometimes necessary to re-treat the cytochrome 550 (1) and 557 (1) fractions with IRC-50 to remove traces of contaminating cytochrome components.

Resolution of fraction AMB 1. Fraction AMB 1 had considerable absorption at 260 mμ. This was almost entirely removed by treating the solution dropwise with 1% (w/v) protamine sulphate until no further precipitation occurred, centrifuging and passing the supernatant through a column of Dowex-2 (X8) (formate). In a typical case the extinction ratio E_{260}/E_{280} was reduced from 1·7 to 0·68 by this treatment with little loss of cytochrome. The cytochrome components still failed to adsorb on IRC-50 at pH 7, but as the subsequent fractionation at pH 5 was more precise after the treatment described, the latter was retained in the separation procedure. Treatment of the original bacterial extract with protamine sulphate and Dowex-2 decreased the yield of enzyme without affecting the overall separation of enzyme and cytochromes.

A cytochrome component of the Dowex-treated fraction AMB 1 was adsorbed on to Amberlite IRC-50 at pH 5 from 0·05 M-citrate. This component (*Thiobacillus X* cytochrome 553·5) was eluted with 0·1 M-phosphate, pH 7. The unadsorbed material (acidic fraction) contained two cytochrome components with bands in the presence of $Na_2S_2O_4$ at 550 and 557 mμ respectively. Attempts to separate these components by fractional precipitation with acetone or ethanol, by paper electrophoresis and by chromatography on calcium phosphate gel were unsuccessful.

Recovery and purity of thiosulphate-oxidizing enzyme and cytochromes. Table 2 shows the activities and recoveries of enzyme at various stages of the

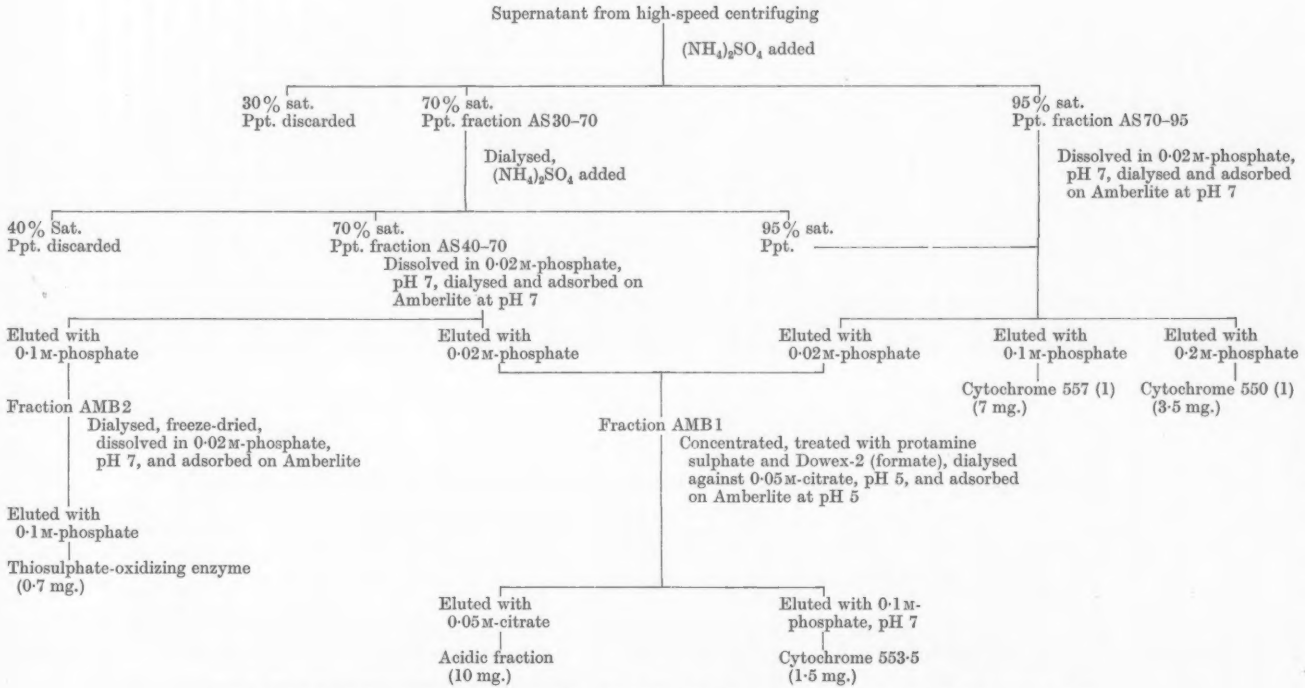
Supernatant from high-speed centrifuging
$(NH_4)_2SO_4$ added
30% sat.
Ppt. discarded
70% sat.
Ppt. fraction AS30–70
95% sat.
Ppt. fraction AS70–95
Dialysed, $(NH_4)_2SO_4$ added
Dissolved in 0·02M-phosphate, pH 7, dialysed and adsorbed on Amberlite at pH 7
40% Sat.
Ppt. discarded
70% sat.
Ppt. fraction AS40–70
95% sat.
Ppt.
Dissolved in 0·02M-phosphate, pH 7, dialysed and adsorbed on Amberlite at pH 7
Eluted with 0·1M-phosphate
Eluted with 0·02M-phosphate
Eluted with 0·02M-phosphate
Eluted with 0·1M-phosphate
Eluted with 0·2M-phosphate
Cytochrome 557 (1) (7 mg.)
Cytochrome 550 (1) (3·5 mg.)
Fraction AMB2
Dialysed, freeze-dried, dissolved in 0·02M-phosphate, pH 7, and adsorbed on Amberlite
Fraction AMB1
Concentrated, treated with protamine sulphate and Dowex-2 (formate), dialysed against 0·05M-citrate, pH 5, and adsorbed on Amberlite at pH 5
Eluted with 0·1M-phosphate
Thiosulphate-oxidizing enzyme (0·7 mg.)
Eluted with 0·05M-citrate
Eluted with 0·1M-phosphate, pH 7
Acidic fraction (10 mg.)
Cytochrome 553·5 (1·5 mg.)

Fig. 2. Flow sheet of procedure for the separation of soluble cytochromes and thiosulphate-oxidizing enzyme. The figures in parentheses refer to the average yield/g. dry wt. of bacteria extracted.

separation procedure. On the basis of extinction at 280 mμ a considerable purification of the crude extract was achieved with a recovery of about 40 %. The final preparation showed no cytochrome bands at −190° and did not fluoresce under ultraviolet light in the presence or absence of thiosulphate.

An assessment of the concentrations of the cytochromes in crude extracts could not be made owing to considerable overlapping of their spectra. This prevented a determination of the recoveries of the individual cytochromes. It was apparent, however, that considerable losses occurred, particularly of cytochrome 553·5, of which only small amounts were obtained. At several stages in the separation procedure insoluble pink precipitates were produced, whereas IRC-50 columns invariably retained pigmented material which resisted elution. No attempts were made in the present study to achieve high degrees of purity for the soluble cytochromes, nor were the various fractions examined by physical tests. However, assuming their molecular weights and α-extinctions in the reduced state to be similar to those of mammalian cytochrome *c*, the best preparations of cytochrome 550 (1) were about 40 % pure and those of cytochromes 553·5 and 557 (1) about 10 % pure.

Properties of the soluble cytochromes

Spectra. At room temperature the reduced cytochromes 550 (1), 553·5 and 557 (1) exhibited typical three-banded spectra between 400 and 600 mμ (Figs. 3–5). All three preparations absorbed strongly in the region of 280 mμ. At the temperature of liquid air the spectra of the reduced soluble cytochromes were much more complex (Table 1) and showed evidence of splitting of the α and β peaks similar to that found with other cytochromes (Keilin & Hartree, 1949; Estabrook, 1956). The main α peaks were at wavelengths 1–4 mμ shorter than those at room temperatures and covered the range visible in intact cells under similar conditions. Indeed, a mixture of cytochromes 550 (1), 553·5 and 557 (1) of approximately equal concentration (based on extinction) showed a spectrum virtually indistinguishable from that of intact cells with the exception of the band at 560 mμ in the latter.

Table 2. *Purification and recovery of thiosulphate oxidase*

For purification procedure and definition of the enzyme unit see text.

Stage of purification	Ratio: Units of enzyme/ml. / $E_{1\,cm.}$ at 280 mμ	Total units
Crude extract	0·5	3900
Fraction AS 40–70	0·85	3470
First IRC-50 treatment	2·1	2150
Enzyme fraction	10·0	1650

Haemochromogens. The main absorption bands of the reduced pyridine and cyanide haemochromogens of the soluble cytochromes are shown in Table 3. Apart from minor variations in the position of absorption maxima the spectra were very similar to those observed when mammalian cytochrome *c* was treated similarly.

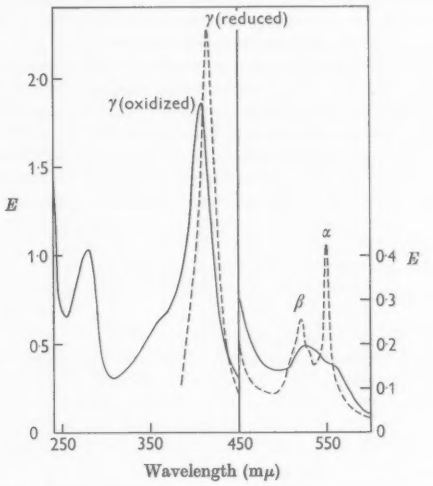

Fig. 3. Absorption spectra of cytochrome 550 (1) (0·55 mg./ml.), oxidized (continuous line) and reduced (broken line). Absorption maxima are: α, 550 mμ; β, 521 mμ; γ (reduced), 416 mμ and γ (oxidized), 409·5 mμ.

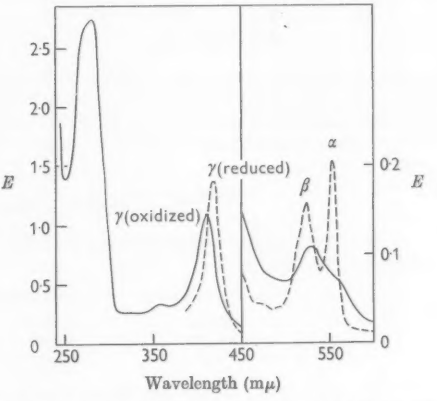

Fig. 4. Absorption spectra of cytochrome 553·5 (2·4 mg./ml.), oxidized (continuous line) and reduced (broken line). Absorption maxima are: α, 553·5 mμ; β, 524 mμ; γ (reduced), 418 mμ and γ (oxidized), 410·5 mμ.

Oxidation–reduction potentials. The standard potentials, E_0' at pH 7 and 30°, were found to be +0·200v for cytochrome 550 (1), +0·210v for cytochrome 553·5 and +0·155v for cytochrome 557 (1).

Effect of carbon monoxide. Carbon monoxide had no effect on the spectra of the reduced soluble cytochromes when freshly prepared or stored at −20° for 2 months. After treatment with acid (pH 3), 2N-NaOH or heating for 3 min. at 60° at pH 7 the α band of all the reduced cytochromes was at 550 mμ. They then reacted with CO, when absorption bands in the reduced state appeared at 560–565 mμ. These changes were also visible after storing the cytochromes for 4–5 days at 2°.

Auto-oxidation. Solutions of reduced cytochrome 557 (1) became oxidized after 1–2 days' storage at 0°. When air was bubbled (30 ml./min.) through a solution of reduced cytochrome 550 (1) it was oxidized at the rate of about 1 %/min. Reduced cytochrome 553·5 was completely oxidized within 5 min. under these conditions. The cytochromes of the acidic fraction were also slowly re-oxidized on shaking or aeration. The addition of cytochrome 553·5 accelerated the auto-oxidation of the other soluble cytochromes. Auto-oxidation of the soluble cytochromes was not inhibited by 0·01M-KCN and was independent of pH within the range 5·5–8·5. After denaturation by acid, alkali or heat all the cytochromes were rapidly auto-oxidized. Addition of denatured cytochrome to untreated samples did not accelerate auto-oxidation of the latter.

Paper electrophoresis. Detailed electrophoretic studies were not undertaken. However, at pH 7 cytochromes 550 (1) and 557 (1) moved towards the cathode whereas cytochrome 553·5 and the components of the acidic fraction moved to the anode, in agreement with their behaviour on Amberlite IRC-50.

Nature of the cytochrome components of the acidic fraction. Apart from their behaviour on Amberlite IRC-50 the components of the acidic fraction had similar properties to those of the separated basic cytochromes 550 (1) and 557 (1). The spectrum of this fraction in the presence of $Na_2S_2O_4$ and at liquid-air temperature showed absorption peaks at 546–549 mμ and at 552–555 mμ (Table 1). Both components were auto-oxidizable, and were denatured by heat, acid and alkali. They did not combine with CO. The main absorption maxima of the cyanide and pyridine haemochromogens of the components of the acidic fraction are listed in Table 3. An approximate estimation of oxidation-reduction potentials was made by the technique described above, except that the extent of reduction was determined with the hand spectroscope by comparison of the bands with those of cytochromes 550 (1) and 557 (1) at different stages of reduction. This indicated potentials, at pH 7, of the order of 0·20v and 0·15v for the 550 and 557 mμ components respectively. No basic components were released

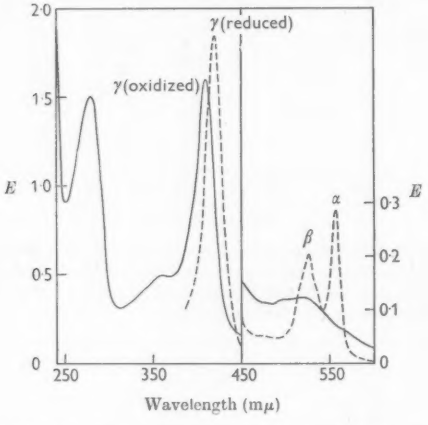

Fig. 5. Absorption spectra of cytochrome 557 (1) (1·4 mg./ml.), oxidized (continuous line) and reduced (broken line). The absorption maxima are: α, 557 mμ; β, 525 mμ; γ (reduced), 419 mμ and γ (oxidized), 409 mμ.

Table 3. *Haemochromogens of soluble cytochromes*

Spectra of the reduced haemochromogens, prepared as described under Materials and Methods, were determined spectrophotometrically.

Cytochrome	Haemochromogen	Absorption maxima (mμ)		
550 (1)	Cyanide	555	525	420·5
550 (1)	Pyridine	550	520	413
553·5	Cyanide	555	524·5	420
553·5	Pyridine	550	521	414·5
557 (1)	Cyanide	556·5	525	420
557 (1)	Pyridine	550	520	413
Acidic fraction	Cyanide	555–557	525	420
Acidic fraction	Pyridine	550	520	413
Mammalian *c*	Cyanide	554	525	420
Mammalian *c*	Pyridine	550	520	414

when the acidic fraction was subjected to the complete separation procedure.

Cytochromes in the particle fractions

Fraction P_1 was almost devoid of cytochrome and thiosulphate-oxidizing enzyme. After reduction with $Na_2S_2O_4$ faint bands at 548 mμ and at 551–555 mμ were observed at liquid-air temperature and were probably due to the small number of intact cells present in this fraction. Fraction P_2 contained no intact cells and in the presence of $Na_2S_2O_4$ absorption bands at 550, 553–558 and 562 mμ were visible at room temperature. A rough comparison with intact cells indicated that about 30–40% of the total cell cytochrome remained in the particles. At liquid-air temperature the particles showed intense absorption at 553–555 mμ and at 547–549 mμ together with faint bands at 560 mμ and between 550 and 553 mμ. The particulate cytochromes were not brought into solution by passage through the Hughes press (Hughes, 1951) nor by treatment with butan-1-ol or detergents.

DISCUSSION

On the basis of the spectra of their haemochromogens the soluble cytochromes described in this paper may be classified tentatively as *c* type. It is now evident that different micro-organisms possess characteristic haem–proteins of the *c* type which differ in various properties from each other and from mammalian cytochrome *c* (cf. Kamen, 1955; Mahler, 1957). Although the cytochrome 553·5 of *Thiobacillus X* closely resembles, in all properties studied, cytochrome c_1 of *Pseudomonas* (Vernon, 1956), both cytochromes 550 (1) and 557 (1) exhibit properties which distinguish them from other *c* cytochromes described in the literature (see review by Morton, 1958).

In Table 4 some properties of the soluble cytochromes of *Thiobacillus X* are summarized and compared with the properties of cytochromes isolated from other thiobacilli. All the cytochromes of *Thiobacillus X* differ in a number of respects from the c cytochrome of *T. denitrificans* and cytochrome *s* of *T. thioparus*. So far only a single cytochrome of the c type has been isolated from each of the last-named two organisms. Indeed, *T. thioparus* has been reported to contain only one c cytochrome (Szczepkowski & Skarzynski, 1952); preliminary observations in this laboratory suggest a similar situation in *T. denitrificans*.

The nature of the two cytochrome components in the acidic fraction is obscure although such properties as have been studied indicate that they are very similar to cytochromes 550 (1) and 557 (1) except for their electrophoretic and ion-exchange behaviour. At first it was thought that cytochromes 550 and 557 may be bound in the cell to acidic molecules and became partly dissociated during separation. However, the stability of the components of the acidic fraction towards treatment with Dowex-2, protamine sulphate and to a further complete fractionation does not support this view.

Alternative views appear to be that either the components of the acidic fraction are two quite distinct cytochromes or that both cytochromes 550 and 557 exist in two forms: one free, the other firmly bound to an acidic molecule. A similar situation exists in *Chlorobium thiosulphatophilum*, from which both a basic and an acidic cytochrome component [*Chlorobium* cytochromes 554 (1) and 554 (2)], with very similar properties, have been isolated (Gibson & Larsen, 1955).

The relationship between the acidic and basic forms will probably be resolved when some knowledge of their function is obtained.

SUMMARY

1. Three soluble cytochromes, *Thiobacillus X* cytochromes 550 (1), 553·5 and 557 (1), and an enzyme system oxidizing thiosulphate, have been separated from extracts of the aerobic sulphur

Table 4. *Properties of c cytochromes from different thiobacilli*

Source	α Band (reduced) (mμ)	E'_0	Absorption on IRC-50 at pH 7	Auto-oxidation at pH 7	Stability to heat	References*
T. denitrificans	552	+0·270	+	−	+	(1)
T. thioparus (cytochrome *s*)	551	+0·140–0·150	−	+		(2)
Thiobacillus X	550	+0·200	+	+	−	
	553·5	+0·210	−	+	−	
	557	+0·155	+	V. slow	−	
Thiobacillus X Acidic fraction	550/557	+0·2	−	+	−	
		+0·15	−			

* (1) Klimek, Skarzynski & Szczepkowski (1956); (2) Aubert, Milhaud, Moncel & Millet (1958).

bacterium *Thiobacillus X*. A fifth fraction (acidic fraction) contained two additional cytochrome components with α bands at 550 and 557 mμ respectively in the reduced state.

2. All the cytochromes appear to be of the *c* type, are labile to heat and acid and do not combine with carbon monoxide. They are auto-oxidizable to varying degrees.

3. Cytochromes 550 (1) and 557 (1) are basic, and are adsorbed on to Amberlite IRC-50 at pH 7. Their E'_0 values at pH 7 are +0·20 and +0·15 v respectively. Cytochrome 553·5 is acidic but is adsorbed on to Amberlite at pH 5. Its E'_0 value is +0·21 v.

4. The cytochromes of the acidic fraction are not adsorbed on IRC-50. Otherwise their properties are very similar to those of cytochromes 550 (1) and 557 (1) respectively.

5. Addition of thiosulphate to whole cells causes a rapid reduction of cytochrome components with α peaks at 549–558 mμ and 562 mμ.

The author thanks Mr P. Davies for skilled technical assistance.

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Thiosulphate Oxidation and Cytochromes in *Thiobacillus X*

2. THIOSULPHATE-OXIDIZING ENZYME

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(*Received* 23 *March* 1960)

The aerobic sulphur autotroph *Thiobacillus X* oxidizes thiosulphate to sulphate with the intermediate formation of tetrathionate (Trudinger, 1959). In the preceding paper (Trudinger, 1961) the separation from extracts of this bacterium of some cytochrome components and an enzyme oxidizing thiosulphate was reported. In this paper some properties of the enzyme system are described and the role of cytochromes in the oxidation of thiosulphate is discussed.

MATERIALS AND METHODS

Thiosulphate-oxidizing enzyme and cytochromes. The preparation of these is described in the preceding paper (Trudinger, 1961).

Enzymic reaction procedures. All reactions were carried out at 23° unless otherwise stated. Ferricyanide reduction was followed with a Unicam SP. 600 spectrophotometer as described by Trudinger (1961). Cytochrome reduction was studied in 5 cm. × 0·4 cm. tubes which were viewed vertically through a Beck microspectroscope, model 3000, mounted on a microscope stage; the condensing lens was used as a source of illumination. Complete reduction of cytochrome was gauged by comparison with a second sample reduced with $Na_2S_2O_4$.

Gas exchanges were measured by the conventional Warburg manometric technique (Umbreit, Burris & Stauffer, 1949).

Radioactive thiosulphate. $Na_2(S{\cdot}SO_3)$, labelled with ^{35}S in either the inner ($-SO_3$) or outer ($-S$) positions, was obtained from The Radiochemical Centre, Amersham, Bucks. Doubly labelled thiosulphate refers to a mixture of equal activities of inner- and outer-labelled thiosulphate.

Potassium tetrathionate. $Na_2S_2O_3,5H_2O$ (50 g.) was dissolved in 100 ml. of water and oxidized to completion with iodine. An excess of potassium acetate was added and $K_2S_4O_6$ precipitated by the addition of 4 vol. of absolute ethanol. The material was recrystallized from 0·5 N-HCl.

Estimation of thiosulphate and tetrathionate. These were determined by titration with standard iodine in the presence of starch before and after reaction with excess of Na_2SO_3 (Kurtenacher, 1924; Starkey, 1934). Ferricyanide and ferrocyanide interfered with the estimation unless the pH of the mixture was kept above 5 and the titration carried out rapidly. A preliminary analysis was carried out to determine roughly the iodine titre. In a second titration slightly less than the required amount of iodine was pipetted into the mixture and the titration completed as rapidly as possible.

Paper chromatography of thiosulphate and tetrathionate. The rear-phase technique of Pollard, McOmie & Jones (1955) was used with Whatman no. 1 paper. Spots were revealed by spraying the paper with 0·5 M-$AgNO_3$ followed by heating at 100° for 2 min. Radioactive areas were detected by exposing the paper to Kodirex X-ray film.

Paper electrophoresis. An apparatus similar to that described by Wood (1954) was used for the electrophoresis of thiosulphate and polythionates. The material to be analysed was spotted 11 cm. from one end (negative) of a 51 cm. × 2 cm. Whatman no. 1 paper strip wetted with 0·05 M-sodium citrate, pH 5·2. The papers were placed in the electrophoresis apparatus under carbon tetrachloride and a voltage of 1000 v was applied for 1 hr. The use of the higher voltage for shorter times than those used by Wood (430 v for 3 hr.) resulted in sharper bands and greater reproducibility. Average mobilities (μ/sec./v/cm.) were 3·65 for thiosulphate and 3·10 for tetrathionate. Some variation in ion movement occurred from day to day owing to changes in room temperature.

Determination of radioactivity. Samples containing less than 1 mg. of dry matter were dried on lens tissue mounted in aluminium or glass planchets. After drying, radioactivity was determined with an end-window Geiger tube. No corrections for self-absorption were necessary under these conditions.

Degradation of radioactive tetrathionate with mercuric chloride. Tetrathionate reacts with $HgCl_2$ according to equation (1) (van der Heijde, 1955).

$$2Na_2[O_3S^{(1)}\cdot S^{(2)}\cdot S^{(2)}\cdot SO_3^{(1)}] + 3HgCl_2 + 4H_2O \rightarrow HgCl_2,2HgS^{(2)} + 2S^{(2)} + 2Na_2SO_4^{(1)} + 2H_2SO_4^{(1)} + 4HCl \quad (1)$$

To 1 ml. of the solution to be analysed was added 0·5 ml. of 40% (v/v) formaldehyde and 0·5 ml. of 5% (w/v) $HgCl_2$ in 2% (w/v) sodium acetate. After 1 hr. at room temperature the mixture was centrifuged and the recipitate was washed twice with 1 ml. portions of 0·01 M-Na_2SO_4. The combined supernatants were then mixed with 20 mg. of inactive $K_2S_4O_6$ and the above described procedure was repeated. The supernatants, containing the sulphate, were combined and made up to standard volume. The precipitates of sulphur and $HgCl_2,2HgS$ were oxidized with conc. HNO_3, saturated with bromine and made to standard volume. Portions of each solution were plated on glass planchets and counted.

Degradation of radioactive tetrathionate with cyanide. Tetrathionate reacts with cyanide according to equation (2) (Brodskii & Eremenko, 1955).

$$Na_2[O_3S^{(1)}\cdot S^{(2)}\cdot S^{(2)}\cdot SO_3^{(1)}] + NaCN + H_2O \rightarrow Na_2[S^{(2)}\cdot SO_3^{(1)}] + NaSCN^{(2)} + H_2SO_4^{(1)} \quad (2)$$

The sample to be analysed (1 ml. of approx. 0·01 M) was made alkaline to phenolphthalein and mixed with 1 ml. of 0·1 M-KCN. After 5 min. at room temperature the products were separated by chromatography on Dowex-2 × 8 (acetate). Sulphate was eluted with 2 M-ammonium acetate, pH 5, thiosulphate with 5 M-ammonium acetate, pH 5, and thiocyanate with 2 N-HNO_3. The identities of sulphate and thiosulphate were checked by electrophoresis on paper and that of thiocyanate by chromatography on Whatman no. 1 paper developed with butan-1-ol–pyridine–water–aq.NH_3 soln. (sp.gr. 0·88) (80:40:80:5; upper phase) (McOmie & Pollard, 1953).

RESULTS

Reaction of thiosulphate-oxidizing enzyme with ferricyanide

Effect of pH. This is shown in Fig. 1. The activity increased markedly as the pH was lowered from neutrality to pH 4·5. Below pH 4·5 a rapid chemical reaction between thiosulphate and ferricyanide, which produced Prussian blue, prevented study of the enzymic reaction. This chemical reaction also occurred at pH 4·5, though at a much slower rate than at pH 4·0, and for this reason pH 5 was chosen for assays of enzyme activity. At pH 6·5 and above a lag occurred in the reduction of ferricyanide; the reason for this is not known. At pH 5, with 5 μ-moles of ferricyanide, the reaction was essentially linear until about 25% of the ferricyanide was reduced.

Effect of thiosulphate concentration. Maximum initial rates of ferricyanide reduction were obtained with concentrations of thiosulphate above about 5 mM (Fig. 2).

Effect of enzyme concentration. The initial rate of ferricyanide reduction increased linearly with enzyme concentration over a 20-fold range (Fig. 3).

Product of thiosulphate oxidation. The product of oxidation of thiosulphate by thiosulphate-oxidizing enzyme could not be separated from authentic tetrathionate by rear-phase chromatography or by paper electrophoresis. Table 1 shows the results of analyses of the product of oxidation of thiosulphate labelled with ^{35}S. When the compound formed from doubly labelled thiosulphate was degraded with $HgCl_2$ the ratio of ^{35}S in the supernatant to that in the precipitate was 1:1·1 compared with the theoretical ratio for uniformly labelled tetrathionate of 1:1. The product of doubly labelled thiosulphate oxidation also reacted with KCN to give sulphate, thiocyanate and thiosulphate. The ratio of ^{35}S in this case was sulphate:thiocyanate:thiosulphate = 1:0·93:2 (theoretical ratio for uniformly labelled tetrathionate, 1:1:2). No

Table 1. *Analysis of the end product of thiosulphate oxidation*

Sodium thiosulphate labelled with ^{35}S was oxidized to completion under similar conditions to those described in Fig. 2. The end product was analysed by reaction with $HgCl_2$ or KCN. Results are expressed as ^{35}S counts/min.

(*A*) $HgCl_2$ reaction

Substrate	End product	Supernatant (SO_4^{2-})	Precipitate ($HgCl_2$,2HgS + S)
$^{35}S - {}^{35}SO_3$	63 000	31 000	34 000
$^{35}S - SO_3$	82 000	50	80 000
$S - {}^{35}SO_3$	46 000	47 000	20

(*B*) KCN reaction

Substrate	End product	Sulphate	Thiocyanate	Thiosulphate
$^{35}S - {}^{35}SO_3$	63 000	15 000	14 000	30 000
$^{35}S - SO_3$	82 000	0	41 000	39 000
$S - {}^{35}SO_3$	46 000	22 000	50	23 000

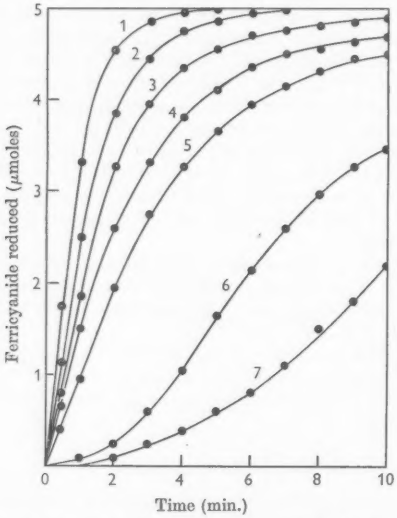

Fig. 1. Effect of pH on the thiosulphate–ferricyanide reaction. The reaction mixtures contained 5 μmoles of $K_3Fe(CN)_6$, 40 μmoles of $Na_2S_2O_3$ and 2·5 units of thiosulphate-oxidizing enzyme in 5 ml. of 0·12 M-buffer (1, phthalate, pH 4·5; 2, phthalate, pH 5·0; 3, phthalate, pH 5·5; 4, phosphate, pH 5·5; 5, phosphate, pH 6·0; 6, phosphate, pH 6·5; 7, phosphate, pH 7·1). Reduction of $K_3Fe(CN)_6$ was followed by changes in E at 420 mμ.

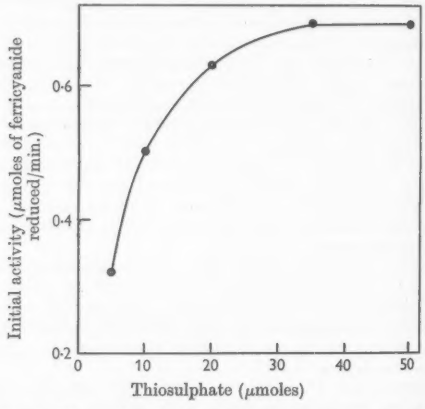

Fig. 2. Effect of thiosulphate concentration on the thiosulphate–ferricyanide reaction. The reaction mixtures contained $Na_2S_2O_3$, 5 μmoles of $K_3Fe(CN)_6$ and 0·6 unit of thiosulphate-oxidizing enzyme in 5 ml. of 0·12 M-potassium phthalate, pH 5.

appreciable exchange occurred between the inner and outer sulphur atoms, which is in agreement with the results obtained with intact cells (Trudinger, 1959).

Stoicheiometry of the thiosulphate–ferricyanide reaction. This is shown in Table 2. The results are in agreement with equation 3.

Reversibility. The thiosulphate–ferricyanide reaction appeared to be irreversible. No thiosulphate was formed when a large excess of tetrathionate was incubated with enzyme and ferrocyanide. Attempts to demonstrate an enzymic exchange of ^{35}S between labelled tetrathionate and thiosulphate in the presence of a catalytic amount of ferricyanide were not successful because of the rapid exchange which occurs spontaneously (cf. Fava, 1953).

Substrate specificity. No reduction of ferricyanide was observed when tetrathionate, trithionate, dithionate or colloidal sulphur replaced thiosulphate under the standard conditions for enzyme assay. Sulphite reduced ferricyanide at the same rate in the presence as in the absence of enzyme.

$$2\,[S{\cdot}SO_3]^{2-} + 2\,Fe(CN)_6^{3-} \rightarrow [O_3S{\cdot}S{\cdot}S{\cdot}SO_3]^{2-} + 2\,Fe(CN)_6^{4-} \qquad (3)$$

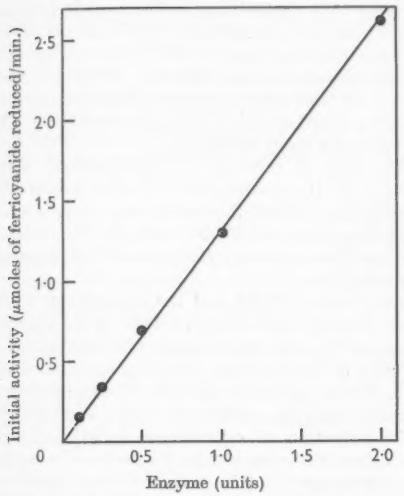

Fig. 3. Effect of enzyme concentration on the thiosulphate–ferricyanide reaction. The reaction mixtures contained thiosulphate-oxidizing enzyme and 40 μmoles of $Na_2S_2O_3$, with other conditions as shown in Fig. 2.

Table 2. *Stoicheiometry of the thiosulphate–ferricyanide reaction*

Enzyme (3·2 units) was incubated with $Na_2S_2O_3$ and $K_3Fe(CN)_6$ in 0·12 M-phthalate, pH 5, until the reaction was complete (no further change in E_{420}). Results are expressed as μmoles.

Expt. no.	Initial		Final		
	$S_2O_3^{2-}$	$Fe(CN)_6^{3-}$	$S_2O_3^{2-}$	$Fe(CN)_6^{3-}$	$S_4O_6^{2-}$
1	5	10	0	5·0	2·55
2	10	5	4·8	0	2·50

Electron acceptors. Methylene blue, phenol blue, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and flavinadenine dinucleotide were not reduced under N_2 by thiosulphate and oxidizing enzyme. Some reduction of dyes of the indophenol series appeared to occur but a chemical reaction between the dyes and thiosulphate masked the reduction process. The reduction of cytochromes will be discussed below.

Effect of some reagents on enzyme activity. Reduction of ferricyanide was not affected by the addition of Mg^{2+} (0·01 M), Mn^{2+} (0·01 M), quinine hydrochloride (2 mM), CN^- (0·01 M), F^- (0·01 M), N_3^- (0·01 M), diethyl dithiocarbamate (1 mM), iodoacetate (1 mM), 8-hydroxyquinoline (1 mM), glutathione (1 mM), pyridoxal phosphate (10 μg./ml.), coenzyme A (CoA; 10 μg./ml.), cocarboxylase (50 μg./ml.) or a boiled extract of *Thiobacillus X*.

Stability of enzyme. Enzyme activity was completely destroyed by heating at 60° for 3 min. at pH 7. About 40% of its activity was lost after 2 months at −25° in 0·02 M-phosphate.

Reaction of thiosulphate-oxidizing enzyme with soluble cytochromes of Thiobacillus X

As only small amounts of material were available, cytochrome reduction was studied with the Beck microspectroscope and the approximate relative rates of reduction were based upon the time taken to reach complete reduction.

Reduction of cytochrome 553·5. Cytochrome 553·5 was reduced by thiosulphate in the presence of the thiosulphate-oxidizing enzyme but not by thiosulphate alone (Table 3). The reduction was more rapid under N_2 than in air, presumably because of the relatively rapid auto-oxidation of this cytochrome (Trudinger, 1961). Potassium cyanide did not increase the rate of cytochrome reduction in air. The anaerobic reaction was not inhibited by CO (saturated) nor by quinine hydrochloride, CN^-, N_3^-, 8-hydroxyquinoline and iodoacetate at the concentrations used previously (see above). No reduction of cytochrome 553·5 occurred when trithionate, tetrathionate or dithionate replaced thiosulphate. Whereas the enzymic reaction between thiosulphate and ferricyanide was maximal in the region of pH 5 (Fig. 1), the rate of reduction of cytochrome 553·5 at this pH was about 10% of the rate at pH 7 (Table 3).

Catalysis of ferricyanide reduction. At pH 7, cytochrome 553·5 catalysed the reduction of ferricyanide by thiosulphate and the thiosulphate-oxidizing enzyme (Fig. 4). The end product was established as tetrathionate by the tests described elsewhere. The reduction of ferricyanide by this system was not affected by the addition of DPN (1 mM), TPN (1 mM), CoA (10 μg./ml.), pyridoxal phosphate (10 μg./ml.) or thiamine pyrophosphate (50 μg./ml.).

In the experiment described in Fig. 4 ferricyanide was reduced at the rate of 0·9 μmole/min./unit of thiosulphate-oxidizing enzyme. This presumably corresponds to the rate of turnover of cytochrome 553·5. The amount of thiosulphate-oxidizing enzyme extracted from 1 mg. of *Thiobacillus X* was about 0·8 unit (Trudinger, 1961), which is equivalent to a rate of cytochrome 553·5 reduction at pH 7 of about 0·7 μmole/min. This is comparable with the rate of oxygen uptake by fresh cells utilizing thiosulphate, which is in the order of 0·3 μg.atom of oxygen/mg. dry wt./min. The unit of thiosulphate-oxidizing enzyme is based on the rate of enzymic reduction of ferricyanide at pH 5 by thiosulphate in the absence of cytochrome (Trudinger, 1961). In the above-mentioned calculation it has been

Table 3. *Reduction of soluble cytochromes by thiosulphate*

Cytochromes were dissolved in 0·1 M-phosphate, pH 7, to give a value for $E_{1cm.}$ at $\alpha_{max.}$ of approx. 0·2. In the experimental mixtures 0·3 ml. of such a solution was used for the major cytochrome component and 0·02 ml. for the minor component. The additions (0·03 ml.) were 0·2 M-$Na_2S_2O_3$ and thiosulphate-oxidizing enzyme (1 unit/ml.). Oxygen-free nitrogen was bubbled through all solutions before and after mixing. The approximate times (min.), for complete reduction of the major cytochrome component, are recorded. No reduction of cytochromes occurred in the absence of $Na_2S_2O_3$.

Cytochrome		Additions	
Major	Minor	$Na_2S_2O_3$	$Na_2S_2O_3$ and enzyme
557	—	∞*	∞
557	553·5	∞	1
557	550	8–9	8–9
553·5	—	∞	< 1
553·5†	—	∞	10–11
553·5	550	7–8	< 1
550	—	8–9	8–10
550	553·5	8–9	1
Acidic fraction	—	7–8‡	8–9‡
Acidic fraction	553·5	7–8‡	1–2‡
Mammalian *c*	—	> 60	15–20
Mammalian *c*	553·5	> 60	15–20

* No observable reduction in 60 min.
† 0·1 M-Phthalate, pH 5, in place of phosphate.
‡ Both components of the acidic fraction were reduced.

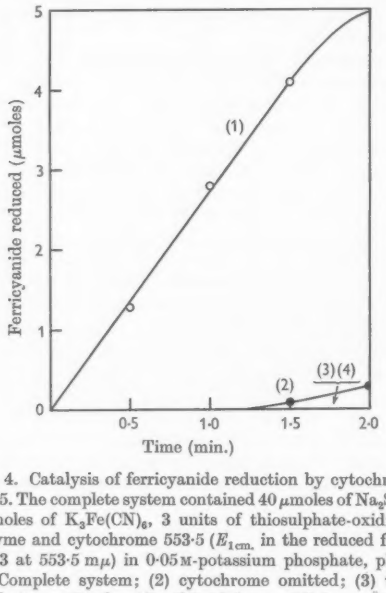

Fig. 4. Catalysis of ferricyanide reduction by cytochrome 553·5. The complete system contained 40 μmoles of $Na_2S_2O_3$, 5 μmoles of $K_3Fe(CN)_6$, 3 units of thiosulphate-oxidizing enzyme and cytochrome 553·5 ($E_{1cm.}$ in the reduced form, 0·023 at 553·5 mμ) in 0·05 M-potassium phosphate, pH 7. (1) Complete system; (2) cytochrome omitted; (3) thiosulphate omitted; (4) thiosulphate-oxidizing enzyme omitted.

assumed that the relation between this rate and that of enzymic cytochrome reduction at pH 7 is the same for both crude and purified preparations of the thiosulphate-oxidizing enzyme. Spectral interference by other cytochromes of *Thiobacillus X* prevented a direct determination of cytochrome 553·5 reduction by crude extracts.

Reduction of other soluble cytochromes. Cytochrome 557 (1) became reduced when mixed with thiosulphate, thiosulphate-oxidizing enzyme and a trace of cytochrome 553·5 (Table 3). The omission of either the enzyme or cytochrome 553·5 prevented this reduction.

Cytochrome 550 (1) and the components of the acidic fraction were slowly reduced by thiosulphate alone and the rates of reduction were unchanged on addition of thiosulphate-oxidizing enzyme. However, in the presence of both thiosulphate and oxidizing enzyme, cytochrome 553·5 catalysed the rapid reduction of cytochrome 550 (1) and the acidic fraction components. The rate of reduction of cytochrome 550 (1) by thiosulphate alone was about the same at pH 5, 6 and 7 and was not decreased by additional chromatography of the cytochrome on Amberlite IRC-50. Cytochrome 550 (1) catalysed a slow reduction of cytochromes 553·5 and 557 (1) by thiosulphate in the absence of the thiosulphate-oxidizing enzyme (Table 3).

After denaturation by heating at 60° for 3 min., none of the soluble cytochromes of *Thiobacillus X* was reduced by thiosulphate with or without the oxidizing enzyme.

The thiosulphate-oxidizing enzyme also catalysed a slow reduction of mammalian cytochrome *c* by thiosulphate (Table 3). This reduction was not stimulated by cytochrome 553·5.

Oxidation of cytochromes and thiosulphate by a particulate system

As described in the preceding paper, the material precipitated from crude extracts of *Thiobacillus X* between 8000 and 145 000 *g* (fraction P_2) contained several cytochrome components. The cytochromes of fraction P_2, prepared from fresh cells, were rapidly reoxidized in air after reduction with a trace of $Na_2S_2O_4$. Fraction P_2 from fresh cells also catalysed the oxidation of the reduced soluble cytochromes 550 (1), 553·5 and 557 (1), the components of the acidic fraction and mammalian cytochrome *c*. In all cases the oxidations were completely inhibited by 0·01 M-KCN.

In the presence of KCN or in the absence of oxygen, the particulate cytochromes became reduced by endogenous substrates within 2–3 min. The particles then showed a spectrum similar to that shown in the presence of $Na_2S_2O_4$ (Trudinger, 1961), with absorption bands in the regions of 550, 552–

Table 4. *Oxidation of thiosulphate by a soluble system and particles*

The manometric flask contained 6·5 units of purified thiosulphate-oxidizing enzyme, cytochrome 553·5 ($E_{1cm.}$ at 553·5 mμ, approx. 0·1), 0·5 ml. of fraction P_2* and 40 μmoles of thiosulphate in 2 ml. of 0·1 M-phosphate, pH 7. The centre well contained 0·2 ml. of 2N-NaOH. Temperature 30°.

System	Oxygen absorbed (μl./60 min.)
Complete	103
Cytochrome 553·5 replaced by cytochrome 550 (1)	45
Cytochrome 553·5 replaced by cytochrome 557 (1)	36
Cytochrome 553·5 replaced by acidic fraction	39
Complete + cytochrome 550 (1) + cytochrome 557 (1)	110
Complete + 0·01 M-KCN	1
Cytochrome 553·5 omitted	40
Enzyme omitted	37
Thiosulphate omitted	35
Fraction P_2 omitted	3

* Particles from 100 mg. dry wt. of fresh cells in 5 ml. of buffer: 10 mg. dry wt. of fresh cells oxidized thiosulphate (under similar conditions) at the initial rate of 1780 μl. of O_2 absorbed/60 min.

558 and 562 mμ. The addition of thiosulphate and thiosulphate-oxidizing enzyme, with or without traces of the soluble cytochromes of *Thiobacillus X*, did not appear to increase the rates of reduction of any of the particulate cytochromes, anaerobically or in the presence of KCN. However, aerobically in the absence of KCN, the 562 mμ-absorption band appeared when fraction P_2 was mixed with thiosulphate, oxidizing enzyme and a trace of cytochrome 553·5.

Thiosulphate was oxidized, with the uptake of oxygen by a system with thiosulphate-oxidizing enzyme, cytochrome 553·5 and fraction P_2 (Table 4). None of the other soluble cytochromes of *Thiobacillus X* could replace cytochrome 553·5 nor did their addition to the complete system increase the rate of oxygen uptake to any marked extent. In the experiment described in Table 4 the complete system oxidized 11·5 μmoles of thiosulphate in 60 min., and 5·6 μmoles of tetrathionate were recovered. The oxygen absorbed, corrected for the amount taken up in the absence of thiosulphate, was 3·0 μmoles, compared with the theoretical value of 2·9 μmoles for oxidation of 11·5 μmoles of thiosulphate to tetrathionate. The rate of oxidation of thiosulphate by this system was low, being about 4% of that of an equivalent amount of intact cells, but no attempt has yet been made to establish optimum conditions for the preparation of active particles.

DISCUSSION

The results presented in this paper indicate that cytochrome 553·5 and the thiosulphate-oxidizing enzyme are involved in thiosulphate oxidation by *Thiobacillus X*. Moreover the amount of enzyme extracted appears to be sufficient to account for the rates of thiosulphate oxidation by intact cells. It is not possible to say whether cytochrome 553·5 reacts directly with thiosulphate-oxidizing enzyme. No evidence was obtained for the presence of an intermediate electron acceptor, but it is probable that both the cytochrome and enzyme preparations were very impure and the possibility of such intermediaries in one or both preparations cannot be excluded.

The particles contain a cyanide-sensitive oxidase for the soluble *Thiobacillus X* cytochromes but otherwise little can be said of the electron-transport chain present in this fraction. On reduction with dithionite the particles exhibit absorption bands in the regions of 550 and 552–558 mμ but spectroscopic studies provided no evidence for the participation of these particulate components in thiosulphate oxidation. The particles also contain a component, with an absorption maximum in the reduced state at 562 mμ, which became partly reduced on the addition of thiosulphate, thiosulphate-oxidizing enzyme and cytochrome 553·5. Should this be a cytochrome of the '*b*' type, as its spectrum indicates, it is unlikely, in view of current concepts of respiratory chains (Chance & Williams, 1956), that it is part of the electron-transport chain between the '*c*' type cytochrome 553·5 and oxygen.

The soluble cytochromes 550 (1), 557 (1) and the components of the acidic fraction equilibrate rapidly with cytochrome 553·5 and are readily oxidized by the particles. They are capable therefore of taking part in electron transfer from thiosulphate to oxygen. The fact that the rate of oxidation of thiosulphate, by the system consisting of thiosulphate-oxidizing enzyme, cytochrome 553·5 and particles, was very much less than the rate of reduction of cytochrome 553·5 indicates that the rate-limiting step was in the particulate electron-transport chain. This probably explains why no stimulation of oxygen uptake was obtained on the addition of cytochromes 550 (1), 557 (1) or the acidic fraction, particularly if a common step is involved in the oxidation of all the soluble cytochromes. The extent to which the various cytochromes are involved in thiosulphate oxidation depends on the relative rates of oxidation and reduction. Such factors cannot be assessed at this stage.

Cytochrome 550 (1) is also reduced by thiosulphate alone but this reaction is too slow to account for much thiosulphate oxidation. It is

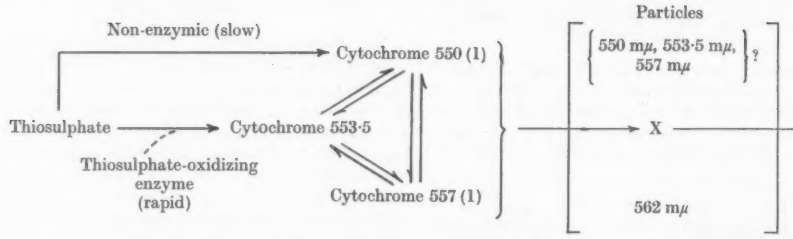

Fig. 5. Summary of electron transport between thiosulphate and oxygen. Arrows indicate the routes of electron transfer demonstrated in this work. The summary is over-simplified to the extent that the cytochromes of the acidic fraction are not included.

unlikely that the cytochrome was contaminated with thiosulphate-oxidizing enzyme for the following reasons: (*a*) the rate of reduction was independent of pH between 5 and 7; (*b*) addition of the separated enzyme did not increase the rate of cytochrome reduction by thiosulphate; (*c*) the rate of reduction was not affected by storage nor by further treatment of the cytochrome with IRC-50. Fig. 5 summarizes the results obtained and indicates possible electron paths during the oxidation of thiosulphate.

The term 'thiosulphate-oxidizing enzyme' has been used in this paper in preference to 'tetrathionase', applied by other workers to enzyme systems converting thiosulphate into tetrathionate (Baalsrud, 1954; Lees, 1955), since the latter term has also been used by Knox & Pollock (1944) for the enzyme system in some heterotrophic organisms, that reduces tetrathionate to thiosulphate. Baalsrud & Baalsrud (quoted by van Niel, 1953) have shown that some heterotrophs are capable of carrying out both the oxidation of thiosulphate and reduction of tetrathionate under aerobic and anaerobic conditions respectively, but there is no evidence that the same enzyme system is involved in both reactions. In the present work no evidence was obtained for the reduction of tetrathionate by the thiosulphate-oxidizing enzyme of *Thiobacillus X*. However, anaerobically, intact *Thiobacillus X* catalyses a rapid conversion of tetrathionate into thiosulphate and other products (P. A. Trudinger, unpublished results). It remains to be seen whether thiosulphate-oxidizing enzyme is involved in this reaction.

SUMMARY

1. Some properties of the thiosulphate-oxidizing enzyme from the aerobic sulphur autotroph *Thiobacillus X* are described.

2. The enzyme catalyses the oxidation of thiosulphate to tetrathionate with ferricyanide or *Thiobacillus X* cytochrome 553·5 as electron acceptors. Other soluble cytochromes from the same bacterium do not react directly with thiosulphate-oxidizing enzyme but become reduced in the presence of thiosulphate, enzyme and cytochrome 553·5.

3. A particulate preparation from *Thiobacillus X* oxidizes the reduced soluble cytochromes 550 (1), 553·5, 557 (1) and the components of the acidic fraction. In the presence of cytochrome 553·5 and thiosulphate-oxidizing enzyme, the particles oxidize thiosulphate to tetrathionate with the consumption of oxygen.

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A Method for Separating Neutral Amino Acids from Neutral Oligopeptides

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(*Received* 3 *May* 1960)

One of the main problems in the isolation of naturally occurring peptides from biological fluids is their separation from the free amino acids which are usually present in relatively large amounts. The separation of amino acids and peptides into groups of basic, acidic, neutral and aromatic compounds (Fromageot, Jutisz & Lederer, 1948) is of some use and Waley (1956), in a study of the acidic peptides of the lens, showed how it was possible to fractionate a complex mixture of acidic peptides once they had been separated from the neutral amino acids. The acidic peptides could readily be separated from the few acidic amino acids by partition chromatography. However, neutral peptides often have such similar chromatographic properties to neutral amino acids that unless they are first concentrated relative to the free amino acids then the latter would swamp them under the usual conditions of partition and ion-exchange chromatography. Precipitation methods with ethanol are not suitable in the isolation of peptides of low molecular weight. Moreover, the grass extracts, for whose fractionation the present study was initiated, contained polyphenolic material, which, if it were precipitated with ethanol, might absorb small peptides. One recent method uses ninhydrin to destroy the free amino acids and leave the unchanged peptide (Markovitz & Steinberg, 1957). The vigorous conditions necessary for this reaction could not be used with confidence in the isolation of naturally occurring peptides of unknown structure. Consden, Gordon & Martin (1946) (cf. Biserte, 1950) separated glycylglycine from glycine by zone electrophoresis at pH 9·2, at which pH the anionic mobility of the peptide exceeds that of the amino acid. This is a useful technique when the proportions of amino acids and peptides are similar but it could not conveniently handle a mixture in which the amino acids were present in many times the concentration of the peptides. For the study of the occurrence of oligopeptides in ryegrass juice it was essential to find a method which would fractionate a few grams of a mixture containing only a small proportion of peptides.

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Synge & Wood (1958) in their studies on bound amino acids in ryegrass obtained a partial separation of peptide-like compounds from amino acids on a column of anion-exchange resin. Their fraction *PVPC* contained practically no bound amino acids, whereas the slightly slower-moving fraction *PWPC* contained a mixture of bound amino acids and free amino acids. They used the resin Dowex-2 (X10) with 0·2 M-sodium acetate. It seemed that this separation might in part be due to the difference in the pK values of the amino groups, causing the peptides to be slightly retarded relative to the free amino acids. Dr S. G. Waley (personal communication) suggested that this effect might be enhanced by using a buffer of higher pH. The pK values for the amino group of the neutral amino acids are all greater than 9·13, and many of the dipeptides and tripeptides of neutral amino acids have pK values less than 8·3 (Cohn & Edsall, 1943). The optimum pH for separation can be calculated from the expression $\frac{1}{2}(pK_{\text{amino acid}} + pK_{\text{peptide}})$ (Davies, 1949). Accordingly, pH 8·65 was chosen, at which value the peptides are predominantly anionic and should be held on an anion-exchange resin, whereas the amino acids are on balance cationic and should pass through the column.

In preliminary experiments at the Rowett Research Institute, such as those described below with ryegrass extracts, such columns were found to pass free amino acids while retaining peptide-like material, and were therefore used as a step in the fractionation schemes of Carnegie (1961) and Synge & Youngson (1961). Later, at Ruakura Animal Research Station, the capabilities of the procedure have been tested with authentic neutral amino acids and dipeptides.

MATERIALS

Anion-exchange resin Dowex-1 (X8) 200–400 mesh/in. (from BioRad Laboratories, 800 Delaware, Berkeley, Calif., U.S.A.), received in the Cl^- form (1·5 kg.), was stirred with 3 l. of 2 N-NaOH for 4 hr. The 'fines' were removed and the resin was washed with water until neutral. It was converted into the Cl^- form by washing

with 4N-HCl, washed with water until neutral and then washed in a column with 4M-ammonium acetate until the effluent was Cl^--free. Before use this resin was equilibrated with the buffer to be used in the experiment.

The amino acids were from California Corp. for Biochemical Research, Los Angeles, U.S.A., and the peptides were from L. Light and Co. Ltd., Colnbrook, Bucks. They were checked for purity by two-dimensional chromatography and ionophoresis (see below). The solutions (1 mg./ml.) were prepared in CO_2-free water.

Buffers. Acetic acid was added to a 25% (w/v) solution of NH_3 to give pH 8·65 and then diluted with CO_2-free water to give a solution in which the acetate concentration was 0·2M. This solution (referred to as 0·2M-buffer) was diluted to give a solution in which acetate was 0·05M and adjusted to pH 8·65 with NH_3 (referred to as 0·05M-buffer). A solution of triethylamine acetate (0·05M-acetate) was prepared in the same way and adjusted to pH 8·5. A 0·25M-triethylamine solution was saturated with CO_2. It was diluted with water to give an approximately 0·05M-solution (pH 8·5). This is referred to as triethylamine carbonate buffer.

METHODS AND RESULTS

Preliminary experiment on separation of amino acids from peptide-like substances in ryegrass extract

The material used in these experiments was obtained by fractionating ryegrass juice according to the procedure of Synge & Wood (1958), to give fraction *PWPC*, which was the second fraction obtained from their Dowex-2 (X10) column with 0·2M-sodium acetate (Dowex-2 is no longer readily available; Dowex-1 is a similar quaternary-ammonium resin). A typical experiment is described below.

Fraction *PWPC* (85 mg. dry matter; N, 14% of dry matter; carboxyl N, 14% of total N before hydrolysis, 55% after hydrolysis) obtained from spring grass cut in 1956 was transferred in a small volume of 0·05M-buffer to a column of Dowex-1 (X8) (30 cm. × 1 cm.) that had been equilibrated with 0·05M-buffer. Elution was started with the same buffer (20–25 ml./hr.) and 35 g. of effluent was collected in fraction 1. Elution with 0·2M-buffer was then started; 35 g. of effluent was collected in fraction 2. 0·1N-HCl was then applied to the column and 22 g. of effluent collected in fraction 3 and 33 g. in fraction 4. (The acetic acid front emerged after 28 g. had been collected from beginning HCl elution.) The 0·1N-HCl was replaced with N-HCl and 27 g. collected in fraction 5. A dark band moved down just before the Cl^- front. After the Cl^- front emerged 15 g. was collected in fraction 6. Fractions were evaporated to dryness *in vacuo* below 40°. The fractions were analysed by the methods used by Carnegie (1961).

The results of this experiment are given in Table 1. Most of the free amino acids were recovered in fractions 1 and 2. Phenylalanine and tyrosine were mainly in fraction 2. The dicarboxylic amino acids were recovered in fraction 4. In fraction 1 there were bound amino acids which could have come from molecules too large to enter the resin particles and be retained. The material in fraction 4 contained a considerable proportion of the total bound amino acids.

Separation of neutral dipeptides from neutral amino acids

A mixture of 1 mg. each of glycine, L-alanine and L-leucine and 0·2 mg. each of glycylglycine, 'DL-alanylalanine' and 'DL-leucyl-leucine' was transferred in 0·05M-ammonium acetate buffer, pH 8·65, to a column of Dowex-1 (X8) (20 cm. × 1·1 cm.). The column was eluted (15 ml./hr.) with 0·05M-buffer and 50 ml. of effluent collected. It was then eluted with M-acetic acid and the effluent collected until the pH of the effluent fell to that of M-acetic acid (30 ml.). The fractions were evaporated to dryness *in vacuo* and the recoveries estimated by the technique described below (Table 2). Within the limits of the technique of

Table 1. *Separation of free from bound amino acids in a fraction from ryegrass on Dowex-1* (X8) *column at* pH 8·65

For details see text. For definitions of abbreviations of amino acids see *Biochem. J.* (1957), **66**, 6.

Fraction	Percentage of carboxyl N taken, recovered in fraction: Before hydrolysis	After hydrolysis†	Major amino acids and other ninhydrin-reacting substances*: Before hydrolysis	After hydrolysis†
Material applied to column‡	100	100	—	—
1. 0·05M-Buffer effluent	80·0	26·8	Gly, Ala, Val, Leu	Gly, Ala, Val, Leu, Phe, Glu
2. 0·2M-Buffer effluent	7·3	23·0	Phe, Tyr, spot near Glu	Gly, Ala, Val, Phe, Leu, Tyr
3. Same as 2	0·0	0·0	None	None
4. Acetic acid front	8·5	24·9	Glu, faint spot between Val and Phe	Gly, Ala, Val, Leu, Phe, Tyr, Asp, Glu
5. Pre-chloride front	10·3	9·2	Glu, Asp, faint spot ahead of Leu	Same as 4
6. Chloride front	0·0	0·5	None	Glu, Asp
Total	106·1	84·4		

* Chromatography in butanol–acetic acid–water (4:1:5, by vol.), Whatman no. 1 paper.
† Hydrolysis with 6N-HCl for 24 hr. at 105°.
‡ Contained (before hydrolysis) 1·65 mg. of carboxyl N, rising to 6·4 mg. after hydrolysis.

estimation all the peptides were recovered in the fraction displaced with acetic acid. The peptides were contaminated with a trace of amino acid. That this did not result from slight hydrolysis of the peptides was demonstrated by a control experiment, in which the peptides alone were run through the column and no free amino acids were detected.

Semi-quantitative technique for estimation of amino acids and dipeptides

A method combining filter-paper electrophoresis and chromatography was used (cf. Biserte, Plaquet-Schoonaërt, Boulanger & Paysant, 1960) and the ninhydrin colours produced were eluted (cf. Thompson & Morris, 1959).

The electrophoresis apparatus was on the principle of Kunkel & Tiselius (1951) with certain modifications to prevent losses and distortion. Two glass plates (43 cm. × 30 cm.) were lightly coated with silicone fluid M.S. 550 (Hopkin and Williams Ltd., London). A sheet of Whatman 3MM paper [washed with 10% (v/v) acetic acid, followed by water until neutral] (53 cm. × 29 cm.) was dipped in 0·026 M-sodium tetraborate (10 g. of $Na_2B_4O_7,10H_2O$/l.) buffer, pH 9·2, and lightly blotted. It was placed between the glass plates with the region where the spots were to be applied prevented from touching the glass by Perspex spacers (cf. Boyd, 1954). The edges and ends were sealed with polythene film coated with grease. The paper was left to equilibrate for 1 hr. before application of the samples. Three samples (two standard mixtures and one unknown) could be run simultaneously on the same sheet if placed at suitable intervals along a line 2·5 cm. from, and parallel to, the longest edge of the paper. Best results were obtained with 50 μg. of each peptide and 25 μg. of each amino acid in a total volume of 50 μl. A potential gradient of 6 v/cm. was applied for 2·5 hr. The paper was then removed, the ends were torn off and it was dried at 70°. It was then suspended in a chromatography tank over both phases of a mixture made from phenol (redistilled) and the above-mentioned tetraborate solution (1:1, v/v). After a few hours the paper was lowered into the phenol phase and developed by ascending chromatography for 11 hr. It was then dried for 5 hr. at 30°. It was dipped briefly into the ninhydrin reagent [2 g. of ninhydrin, 10 ml. of acetic acid, 5 ml. of water, 0·2 ml. of pyridine (British Drug Houses Ltd., technical grade) and 85 ml. of acetone] and heated at 70° in an oven (atmosphere saturated with ethanol vapour) for 30 min. A typical chromatogram is shown in Fig. 1. The spots were cut out under subdued light and immediately placed in 5 ml. of a mixture of methanol (500 ml.) and saturated aqueous cupric nitrate (0·2 ml.) (Zaĭtseva & Tyuleneva, 1958) and shaken in the dark for 30 min. The copper changes the purple ninhydrin colour to a more stable red colour. Absorption was read at 540 mμ after decanting the solution into colorimeter tubes and allowing any lint to settle. Colours were stable for at least 2 hr. As with most estimations of this type, it is advisable to run standard samples on the same sheet to allow for any variation in colour production.

Control experiments indicated that standard quantities of amino acids (25 μg.) and peptides (50 μg.) estimated by this technique gave colours whose extinctions had the following coefficients of variation: leucine 7·5, alanine 1·3, glycine 2·5, leucyl-leucine 2·7, alanylalanine 0·9, glycylglycine 4·5.

Ammonium acetate is not a very volatile buffer. A column was run with the more volatile 0·05 M-triethylamine acetate buffer, pH 8·5. Similar recoveries to those shown in Table 2 were obtained.

As an additional check, the recovery of glycylglycine from a column was estimated with the ninhydrin–CO_2 procedure of Van Slyke, Dillon, MacFadyen & Hamilton (1941) as modified by Synge (1951).

Glycylglycine (1 mg.) was transferred to a column of Dowex-1 (X8) (25 cm. × 1·1 cm.) in the acetate form with 0·05 M-ammonium acetate buffer. The column was operated as already described. The fractions, after evaporation to dryness, were hydrolysed in 6 N-HCl at 105° for 24 hr. and the carboxyl N was determined. Glycylglycine (1 mg.) was similarly hydrolysed and the carboxyl N estimated.

The recovery of glycylglycine in the fraction displaced by M-acetic acid was 101%. There was a slight evolution of CO_2 from the reaction with the hydrolysate of the 0·05 M

Table 2. *Amino acids and peptides recovered in fractions from Dowex-1 (X8) with ammonium acetate buffer*

For details see text.

Amino acid or peptide	Percentage of amount taken in mixture: 0·05 M-Buffer eluate	Percentage of amount taken in mixture: M-Acetic acid eluate
Leucine	94	<5*
Alanine	90	<5*
Glycine	99	<5*
Leucyl-leucine	0	103
Alanylalanine	0	101
Glycylglycine	0	101

* Quantities present were too small for accurate determination by ninhydrin technique.

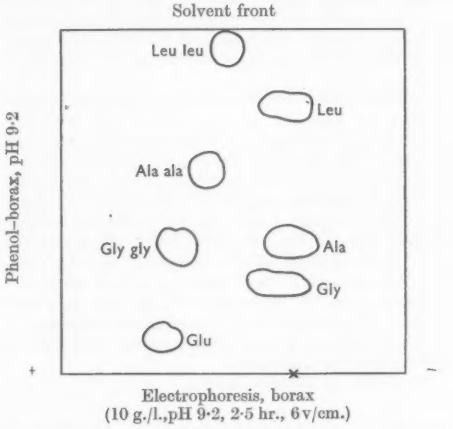

Fig. 1. Two-dimensional separation of amino acids and peptides on filter paper (see text). Ionophoresis horizontal (× indicates point of application of mixture), 2·5 hr., 0·026 M-sodium tetraborate, pH 9·2, 6 v/cm. Chromatography upwards (phenol–0·026 M-sodium tetraborate, pH 9·2) (× 0·23).

Table 3. *Recovery of glycine from Dowex-1 (X8) as percentage of glycine taken*

For details see text.

Fraction eluted with	Percentage recovery: Resin and buffer in acetate form	Percentage recovery: Resin and buffer in carbonate form
0·05 M-Buffer, pH 8·5	98	34·2
M-Acetic acid	2·2	63·3

fraction, possibly caused by the presence of a large amount of ammonium chloride.

Formation of carbamino acids

The presence of traces of free amino acids in the peptide fraction could not at first be explained. They were not removed by increasing the volume of effluent collected in the 0·05 M-fraction. Anion-exchange resins, especially in the higher pH regions, can exert a slight cation-exchange capacity (Partridge & Brimley, 1949), which might explain the slight retention of the free amino acid on the resin. Alternatively, and more likely, the retention might be due to traces of CO_2 converting the amino acids into the dicarboxylic carbamino acids, which would be held on the resin. As a check on this hypothesis, two columns were run under the same conditions except that in one the resin was in the acetate form and in the other it was in the carbonate form.

A column of Dowex-1 (X8) (20 cm. × 1·1 cm.) was equilibrated with 0·05 M-triethylamine acetate buffer, pH 8·5, with precautions to exclude CO_2. Glycine (10 mg.) in 1 ml. of CO_2-free water was applied to the column. The column was eluted with 50 ml. of buffer and the first fraction collected. Then it was washed with 30 ml. of M-acetic acid and the second fraction collected. The carboxyl N content of each fraction was determined after evaporation to dryness and leaving overnight in a vacuum desiccator.

A column of Dowex-1 (X8) in the acetate form (20 cm. × 1·1 cm.) was converted into the carbonate form by prolonged washing with approx. 0·25 M-triethylamine carbonate, pH 8·5. The column was then equilibrated with 0·05 M-triethylamine carbonate buffer. Glycine (10 mg.) in 1 ml. of CO_2-free water was applied to the column and eluted with 50 ml. of buffer, being collected in the first fraction. The resin was then transferred to a beaker and stirred with 30 ml. of M-acetic acid until evolution of CO_2 ceased. The slurry was returned to the column and washed with 35 ml. of M-acetic acid. The combined washings were the second fraction. After evaporation of the mixtures to dryness and leaving overnight in a vacuum desiccator the carboxyl N in each fraction was determined.

The results are given in Table 3.

DISCUSSION

Anion-exchange resins have been used in a similar manner to that described above for other separations. A difference between the pK values of the amino group of leucine and methionine was made use of by Davies, Hughes & Partridge (1950) to achieve a separation on a column of Dowex-2 by running an aqueous solution of the amino acids into the resin and then displacing them with hydrochloric acid. Similar procedures are coming into general use in work with nucleotides. Thus, for example, cytidine 2′:3′-phosphate was purified on a column of Dowex-1 with triethylamine acetate buffer, pH 7·4 (Crook, Mathias & Rabin, 1960). Synge & Youngson (1961) used the method described here, in their studies on the incorporation of L-[^{14}C]valine into non-protein nitrogenous fractions of ryegrass, to separate bound valine present in amphoteric peptide-like compounds from free amino acids. In their control experiments only 0·3% of free L-[^{14}C]valine applied to the column was retained on the resin. The properties of the bound amino acids retained on the resin are discussed by Carnegie (1961). It is possible that the presence of glutamic acid and aspartic acid residues with free carboxyl groups in some peptides aided in their retention on the resin.

Even with precautions to exclude carbon dioxide, small amounts of amino acids remained on the resin and were displaced with the peptides, which is a disadvantage if a complete separation were required. However, after removal of the majority of the amino acids the remainder can easily be removed by electrophoresis at pH 9·2 with a volatile-buffer system (cf. Carnegie, 1961). Care has to be taken that the flow rate of the buffer is not too fast and that the column is not overloaded. The exchange capacity of anion-exchange resins is low in the region pH 8–9 (Partridge & Brimley, 1949).

Formation of carbamino acids. This possible explanation for retention of free amino acids by the resin was suggested in a personal communication by Dr J. A. Mills (Adelaide). The results shown in Table 3 support the view that in the presence of carbonate there was a fairly rapid conversion of glycine into its carbamino derivative, which was retained on the resin. Leucine gave similar results.

There have been surprisingly few studies on this reaction of carbon dioxide with amino acids, which may be important in biological reactions, not only as a mechanism for carbon dioxide fixation but also in protein synthesis. The early work (for references see Siegfried, 1907) on the formation of carbamino acids was carried out in highly alkaline solutions and until recently it seems to have been assumed that the reaction takes place only at high pH. Recently, however, it has been shown that, in the presence of bicarbonate, carbamino acids can be formed at neutral and even slightly acid pH values (Neuberg, Grauer & Kreidl, 1955). Giustina & Temelcou (1955) have studied the formation of carbamino acids at different pH values and found

that the amount of carbon dioxide bound by glycine in aqueous solution at equilibrium reaches a maximum at pH 9·5, whereas glycylglycine and diglycylglycine bind the maximal amount at pH 8–9. Also these peptides have five times as great an affinity for carbon dioxide as has glycine.

It is interesting to speculate on the role that carbamino acids may play in amino acid activation and protein biosynthesis. There may be an enzyme system in the cell which would rapidly remove the carbamino acid driving the equilibrium reaction

$$\begin{matrix} R{-}NH_2 \\ | \\ CO_2^- \end{matrix} + H{\cdot}CO_3^- \rightleftharpoons \begin{matrix} R{-}NH{\cdot}CO_2^- \\ | \\ CO_2^- \end{matrix} + H_2O$$

from left to right in a manner analogous to the trapping of the dicarboxylic carbamino acid on the anion-exchange resin. The low-energy zwitterion is changed by the addition of carbon dioxide into a compound with higher energy. Wieland & Pfleiderer (1957) speculated on the role of carbamino acids in peptide synthesis. They suggested that carbon dioxide may also have a biological role in protecting the amino groups, analogous to the use of benzyl chloroformate in synthetic peptide chemistry.

SUMMARY

1. Monocarboxylic monoamino acids have been separated from their dipeptides on a column of anion-exchange resin eluted with ammonium acetate buffer, pH 8·65. The peptides are retained on the resin and later eluted with acetic acid.

2. A semi-quantitative technique with two-dimensional chromatography–ionophoresis on filter paper was devised for assessing the results of these separations.

3. The presence of carbon dioxide interfered with the separations and it is suggested that this was due to formation of carbamino acids. The possible role of such compounds in the biosynthesis of proteins is discussed.

4. With juice expressed from ryegrass the procedure was useful for removing free amino acids from peptide-like compounds of low molecular weight which it was desired to study in greater detail.

I thank Dr J. A. Mills, Dr R. L. M. Synge and Dr S. G. Waley for advice and encouragement. Part of this work was done while holding an Agricultural Research Council Studentship. This work forms part of a Ph.D. Thesis to be submitted to the University of Aberdeen.

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Biochem. J. (1961) **78**, 692

Filter-Paper Ionophoresis of Cupric Complexes of Neutral Amino Acids and Oligopeptides

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(*Received* 3 *May* 1960)

We did these experiments in the hope of developing a general procedure for separating dipeptides from higher oligopeptides. Dobbie & Kermack (1955*a*, *b*; cf. Dobbie, Kermack & Lees, 1955) have given evidence that tripeptides form different types of complex with cupric ions from those formed by dipeptides. This could be the basis for an ionophoretic group separation. Our preliminary experiments were encouraging and we have now made a detailed comparison of the ionophoretic behaviour of the cupric complexes of glycine, glycylglycine and diglycylglycine with that predicted from the equilibrium constants given by Kermack and colleagues. The agreement is good. Triglycylglycine (whose equilibrium constants are not available in the literature) behaved similarly to the tripeptide under our conditions, so the method has possibilities for group separations of naturally occurring mixtures.

Kermack and colleagues used filter-paper ionophoresis for testing their theory of complex formation with glycylglycine, but not with diglycylglycine. Our results with the latter compound provide an independent line of evidence on the reaction of cupric ions with the tripeptide. Moreover, whereas their experiments were done with a limited amount of cupric ion migrating in a buffer containing the ligand at fixed concentration, we have studied the migration of a limited amount of ligand in a buffer solution saturated with cupric ion.

We gave a preliminary account of this work to the New Zealand Institute of Chemistry (Carnegie & Synge, 1959) and at the Parkville Symposium on Proteins (cf. Thompson, 1959).

MATERIALS

Glycine and glycylglycine were obtained from British Drug Houses Ltd., Poole, Dorset. Diglycylglycine was from L. Light and Co. Ltd., Colnbrook, Bucks. Triglycylglycine was synthesized from diglycylglycine with *p*-nitrophenyl bromoacetate on the suggestion of Dr D. W. Russell (see Addendum). The resulting bromoacetyl derivative was not characterized but aminated as for the corresponding chloro compound (Fischer, 1904). *N*-2:4-Dinitrophenylethanolamine was prepared according to James & Synge (1951).

METHODS

The paper-electrophoresis apparatus of Kunkel & Tiselius (1951) was used. The glass plates (45 cm. × 20 cm.) were smeared lightly with Silicone Fluid 550 (Dow Corning Corp., Midland, Mich., U.S.A.). A solution of 0·026M-sodium tetraborate (10 g. of $Na_2B_4O_7,10H_2O$/l.) and 0·0046M-sodium phosphate (1·7 g. of $Na_2HPO_4,12H_2O$/l.) was used as the buffer (pH 9·2). A suspension of copper phosphate in 2% (w/v) sodium tetraborate was prepared according to Martin & Mittelmann (1948). Whatman 3MM paper was dipped into the buffer, which had been saturated with copper phosphate by the addition of copper phosphate suspension (4 ml./l.). After being lightly blotted, the paper was left for a few hours in the apparatus to equilibrate with buffer. No copper phosphate suspension was added to the buffer used for the runs with the free amino acids and peptides. A potential gradient of 7·5v/cm. was applied for 4½ hr. The runs were made at room temperature with no special arrangements for temperature control. Solutions of the amino acids and peptides used were 0·05M in the buffer. Copper phosphate suspension (0·2 ml.) was added to 1 ml. of the above-described solutions to form the copper complexes. The excess of copper phosphate was allowed to settle. Each solution (20 µl.) was applied to the starting line after the equilibration period. A saturated solution of *N*-2:4-dinitrophenylethanolamine was used as a marker of electroendosmosis; this was not appreciably adsorbed on the paper and did not seem to form a complex with borate. After the run the papers were dried at 105°.

Spray reagents. A 0·2% solution of ninhydrin in water-saturated butanol, to which was added about 2% (v/v) of acetic acid, was used to show the amino acids and peptides. A 0·1% solution of rubeanic acid (dithio-oxamide, British Drug Houses Ltd.) in aqueous 90% (v/v) acetone was used to show the position of copper.

Solubility product of copper phosphate. A value for the solubility product of copper phosphate could not be found in the literature. An approximate value was determined by estimating the concentration of copper in the supernatant of a saturated solution of copper phosphate in the buffer after prolonged centrifuging. Copper phosphate suspension (5 ml.) was mixed with 30 ml. of the above-described buffer and centrifuged for 30 min. at 2000 rev./min. (500 *g*). The

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supernatant was discarded and the precipitate stirred with 40 ml. of buffer and centrifuged for 8 hr. in a covered tube at 2400 rev./min. (720 *g*). The copper content of the supernatant, determined by the method of Clare, Cunningham & Perrin (1945), was found to be $10^{-5\cdot5}$M (g.atom/l.). This is compatible with values calculated from the blank determinations of Martin & Mittelmann (1948), Blackburn & Robson (1953) and Borchers (1959), who used slightly different supernatants. Albert (1951) gives evidence that there is no interaction of cupric ions with borate.

RESULTS

Table 1 shows the mobilities on paper of the cupric complexes of glycine and its peptides and of the free ligands after correcting for electroendosmosis, which was in the direction of the cathode. No correction was made for any adsorption on the paper. Glycine had a slight anionic mobility at pH 9·2 but in the form of its cupric complex it had a slight cationic mobility. The copper–glycine spot had a sharp leading edge. The mobilities of the free tripeptide and the copper complex of the tripeptide are similar, but the mobility of the copper complex of the dipeptide is much less than the mobility of the free dipeptide. The copper complexes of glycine and glycylglycine were blue in colour and that of diglycylglycine was violet. The 0·05M-solution of triglycylglycine gave a blue copper complex. When this solution was diluted with buffer on the paper it gradually changed to a violet colour similar to that of the tripeptide. The blue complex moved anionically at a slower rate than the violet complex, which thus appeared first as a streak on the anode side of the blue spot. As the run proceeded all the blue complex changed to the violet form, which had a mobility similar to that of the tripeptide–copper complex. The blue and violet tetrapeptide complexes changed to a rose colour when the pH was raised by spraying with aqueous NaOH. A mixture of the copper complexes of these peptides and glycine separated into three groups. The diglycylglycine–copper and triglycylglycine–copper complexes did not separate.

From the dissociation constants given by Dobbie *et al.* (1955) and Dobbie & Kermack (1955*a*, *b*) the proportions of the different molecular species expected to be present at pH 9·2 were calculated. The results are shown in Table 2. In other experiments, conducted with the same methods, similar behaviour was observed for the cupric complexes of leucine, leucylglycine and leucylglycylglycine, and for the cupric complexes of alanine and alanylalanine.

Table 1. *Ionophoretic mobilities on filter paper at* pH 9·2

For conditions see text. +, Anionic mobility; −, cationic mobility.

Ligand	Mobility of cupric complex (cm².v⁻¹.hr.⁻¹)	Mobility of free ligand (cm².v⁻¹.hr.⁻¹)
Glycine	−0·03	+0·10
Glycylglycine	+0·24	+0·44
Diglycylglycine	+0·39	+0·39
Triglycylglycine	+0·39	+0·34

DISCUSSION

Our results for the mobilities of the cupric complexes of glycine, glycylglycine and diglycylglycine seem consistent with those that would be expected from the distribution of molecular species calculated from the equilibrium constants of Kermack and colleagues (Table 2), if one assumes instantaneous equilibration and the usual relationship between hydrodynamic drag and molecular bulk for each species of complex present. These constants are in agreement (so far as the present experiments are concerned) with the data of Datta & Rabin (1956*a*) (cf. Manyak, Murphy & Martell, 1955; Rabin, 1956; Datta & Rabin, 1956*b*; Weiss & Fallab, 1957; Murphy & Martell, 1957; Biester & Ruoff, 1959). Koltun & Gurd (1959) and Koltun, Fried & Gurd (1960) have also provided quantitative corroboration of the reaction scheme of Kermack and colleagues. Some of these workers doubted whether existing evidence justified Kermack and colleagues in postulating ionizing copper–$(peptide)_2$ complexes. However, the amounts of these formed under our conditions should in any case have been negligible.

As concerns the cupric (M) complexes of free glycine, calculations for increasing dilutions of ligand (L) show an increasing proportion of ML^+ which would migrate cationically; at still higher dilutions L^- and L^{+-} not in a complex, with anionic migration, would predominate. This serves to explain both the cationic migration of the spot and the sharpness of its leading edge. These changes in distribution of molecular species with decreasing concentration of total ligand also serve to explain divergences from stoicheiometry when small amounts of free amino acids are determined under conditions resembling those of Pope & Stevens (1939). Mixtures of ML^+, L^- and L^{+-} could here also bear a spurious resemblance to ML_2 in their stoicheiometry. Divergences have been noted by a number of workers, especially at higher phosphate concentrations in the buffer used, which result in lower [Cu^{2+} ion] (Kober & Sugiura, 1913; Martin & Mittelmann, 1948; Woiwod, 1949; Schroeder, Kay & Mills, 1950; Blackburn & Robson, 1953; Boser, 1954; Spier & Pascher, 1954; Beauchene, Berneking, Schrenk, Mitchell & Silker, 1955; Baudet & Cherbuliez, 1955; Hagan, Ablondi & Hutchings, 1956; Sobel.

Table 2. *Calculated percentage distribution of ligand between different molecular species present at* pH 9·2

In all cases the proportion of L^+ is negligible. For glycylglycine and diglycylglycine at 0·0005 M the proportions present as different complexes are closely similar to those at 0·05 M except that the complexes involving 2 molecules of ligand (marked*) are present in even smaller proportions.

Ligand	Total concn. of ligand (M)	In the presence of Cu^{2+} ion ($10^{-5.5}$ M)								In the absence of Cu^{2+} ion	
		L^-	L^{+-}	ML^+	ML	ML^-	$ML_r(OH)^-$	ML_2	ML_2^-	ML_2^{2-}	L^-	L^{+-}
Glycine	0·05	0·003	0·015	6·1	—	—	—	93·9	—	—	18	82
Glycine	0·0005	0·034	0·155	37	—	—	—	63	—	—	18	82
Glycylglycine	0·05	0·0004	—	0·0008	73·8	—	26·2	—	0·03*	0·003*	87	13
Diglycylglycine	0·05	0·0001	0·00001	0·00006	0·6	99·4	—	—	0·00012*	0·0009*†	91	9

* Complex involving 2 molecules of ligand.
† Aggregate of symmetrical and asymmetrical species.

Henry, Chiamori & Segalove, 1957; Borchers, 1959). A systematic study of the dependence of these divergences on concentration, both for free amino acids and for some oligopeptides, has been made by Rauen, Leonhardi & Buchka (1949).

Our calculations show that with glycylglycine and diglycylglycine only 1:1 cupric complexes are to be expected in appreciable quantity and the proportions of the main species present should not be much affected by dilution. The formation of only 1:1 complexes precludes the existence of mixed complexes of one atom of copper with two different ligands, which would interfere with separations of mixtures and does in fact interfere with separations of free amino acids. We have satisfied ourselves about the absence of interaction of the cupric complexes of the peptides and interaction of those of free amino acids (cf. Synge, 1956), with the continuous-electrophoresis procedure of Grassmann & Hübner (1953) (see also Pope & Stevens, 1939; Bailey, Chibnall, Rees & Williams, 1943; Schroeder *et al.* 1950; Bennett, 1959). Evidence for formation of 1:1 complexes of copper with peptides under a wide variety of conditions has been presented by Kober & Sugiura (1912, 1913), Kober & Haw (1916), Kerkkonen (1948), Rauen *et al.* (1949), Woiwod (1949), Spies & Chambers (1951), Baudet & Cherbuliez (1955), Buděšínský (1957) and Borchers (1959). Rising, Parker & Gaston (1934), Wenaas (1937), Manyak *et al.* (1955), Buděšínský (1957) and Rosenberg (1957) have isolated in crystalline form copper–peptide complexes possessing this stoicheiometry. Li & Doody (1954), however, interpreted their results as implying that glycylglycine forms only a 1:2 complex with Cu^{2+} ion. Abderhalden & Fodor (1912), Abderhalden & Schnitzler (1927) and Feldman (1937) isolated cupric complexes of various peptides of both the above types. Sauerwein (1937) isolated complexes with more than 1 atom of copper to 1 molecule of peptide. Plekhan (1951*a*, *b*) has obtained spectrometric evidence for the formation of mixed cupric complexes of glycylglycine with diglycylglycine and triglycylglycine at high pH values. Our own observations on triglycylglycine suggest that at pH 9·2 it may exist largely as a complex with more than 1 molecule of peptide/copper atom (blue) passing over on dilution to a violet 1:1 complex of higher anionic mobility. The spectrometric studies of Kober & Haw (1916) on the cupric complexes of a number of tetrapeptides at different pH values support this view. The observations of Dobbie & Kermack (1955*b*) on the behaviour of the tripeptide complexes at different concentrations and pH values suggest similar behaviour.

At pH 9·2 our procedure should be generally useful for separating neutral dipeptides from tri-

peptides and perhaps from higher peptides. Experience with leucylglycine and leucylglycylglycine supports this view. The separation of oligopeptides from free amino acids is scarcely favoured by the use of cupric complexes. The differences in mobility of the free compounds at pH 9·2 due to differences in the pK values of their amino groups are equally great (Consden, Gordon & Martin, 1946), and separation on anion-exchange resin at pH 8·6 is also a useful procedure (Carnegie, 1961). The colours of the cupric complexes, which are easily seen, are useful for following the course of fractionations. There should be no difficulty in regenerating peptides from their cupric complexes with hydrogen sulphide; that encountered by Tomita, Hamamura, Tamiya, Takehara & Tomita (1953) is perhaps explained by the polymorphism of glycylglycine (Bernal, 1931).

SUMMARY

1. The ionophoretic behaviour of glycine, glycylglycine, diglycylglycine and triglycylglycine has been studied at pH 9·2 in the presence and absence of a fixed concentration of free cupric ion.

2. The behaviour of the cupric complexes of the first three compounds mentioned is consistent with that to be expected from the schemes of reaction and equilibrium constants given by Dobbie & Kermack (1955*a*, *b*) and by Dobbie *et al.* (1955).

3. The observations made are discussed in relation to the literature on the cupric complexes of amino acids and peptides. They suggest the basis for a group separation of dipeptides from higher oligopeptides.

We wish to thank Dr H. C. Freeman and Professor W. O. Kermack for helpful discussions, Dr S. Davidson for help with the calculations and Mr M. R. Coup for copper determinations. One of us (P.R.C.) is grateful to the Agricultural Research Council for a Studentship. The experimental part of this work forms part of his Ph.D. Thesis to be submitted to the University of Aberdeen.

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ADDENDUM

Acylations with Esters of *p*-Nitrophenol

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(*Received* 3 *May* 1960)

The purpose of this Addendum is to draw attention to the potentialities of *p*-nitrophenyl esters as acylating agents for amino acids in aqueous solution.

Satisfactory acylation of diglycylglycine with *p*-nitrophenyl bromoacetate is reported in the paper above, after poor results had been obtained with chloroacetyl chloride. The technique used was one developed for peptide synthesis, in which acylation of free amino acids with *p*-nitrophenyl esters of benzyloxycarbonyl amino acids is carried out in unbuffered solution with a high initial pH (Elliott & Russell, 1957; Russell, 1959). Reaction in neutral solutions is much slower, and low yields have been reported (Bodánszky, 1957; Farrington, Hextall, Kenner & Turner, 1957).

Yields of chloroacetyl amino acids obtained by Schotten–Baumann or direct acylation with chloroacetyl chloride (Ronwin, 1953) are usually only moderate and sometimes poor. In such cases better results may be obtainable with a *p*-nitrophenyl haloacetate, as in the present instance. Esters of *p*-nitrophenol are stable, crystalline solids which are easy to prepare (Elliott & Russell, 1957; Bodánszky & du Vigneaud, 1959). As such they offer advantages over the more reactive acyl halides and should be considered as alternative reagents in cases where the acyl halides prove unsatisfactory.

MATERIALS AND METHODS

p-Nitrophenyl bromoacetate prepared from *p*-nitrophenol and bromoacetyl bromide (cf. Speroni, Losco & Peri, 1951) and recrystallized from *cyclo*hexane, had m.p. 83–84° (corr.) [Found: *p*-nitrophenol released on hydrolysis (Schwyzer & Gorup, 1958), 53·5; $C_8H_6BrNO_4$ requires: *p*-nitrophenol, 54·0%].

Technique of acylation. The amino acid or peptide (1 mole) and Na_2CO_3 ($\frac{1}{2}$–1 mole) were dissolved in N-NaOH (1 equiv.) and a solution of a *p*-nitrophenyl ester (1 mole) in *NN*-dimethylformamide was added with vigorous shaking, which was continued for $\frac{1}{2}$–1 hr. The reaction mixture was diluted with water and filtered, and the acylated product precipitated by acidification.

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Bound Amino Acids of Ryegrass: The Isolation of Amphoteric Peptide-like Substances of Low Molecular Weight

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(*Received* 3 *May* 1960)

Although grass is the most important foodstuff for ruminant and other herbivorous livestock, remarkably little is known about the nature of its non-protein nitrogenous compounds, other than the free amino acids. Although peptides of low molecular weight account for only a small percentage of the non-protein nitrogen, they may be important in the nutrition of rumen micro-organisms and of micro-organisms involved in the making of silage. More information on these compounds is desirable for a better understanding of plant physiology in general and for the mechanism of protein synthesis in particular. There have been few studies on the isolation of plant peptides but many qualitative observations on the differences between the amino acid pattern on chromatograms of plant extracts, before and after hydrolysis. This chromatographic evidence was usually unsupported by other observations and on its own was insufficient to justify the claim that the unidentified substance was a peptide. The aim of the present work was to isolate peptides of low molecular weight from the non-protein nitrogenous fraction of ryegrass, in other words, those amphoteric compounds with a molecular weight under 10 000 composed only of amino acids linked together by peptide bonds. In the course of their studies on neutral and acidic substances containing bound amino acids in ryegrass Synge & Wood (1958) isolated a fraction (*PWPC*) which could have contained genuine peptides.

There are several major difficulties in the isolation of such peptides. They are present in such low concentrations that large amounts of grass must be fractionated in order to get a final yield of a few milligrams. Since oligopeptides are similar in their physical properties to their component amino acids and also because of their low concentration relative to the free amino acids, it is difficult to achieve a separation of the peptides from the amino acids by the usual chromatographic and electrophoretic procedures. The fractionation scheme used in the present work (see Fig. 1) was designed to collect as large a proportion as possible of any neutral oligopeptides free from amino acids, strongly acidic and basic peptides and other non-protein nitrogenous compounds.

In the first stage the cell membranes were plasmolysed by treatment with ether (Chibnall, 1939) and the juice was squeezed out. The expressed juice contained little protein, since the cell wall acted as a filter. The cation-exchange resin not only rejected uncharged compounds and acids, but also by its molecular-sieve action rejected proteins and peptides of high molecular weight (cf. Waley, 1957). The amino acids, peptides and strong bases were retained on the resin, but only the amino acids and peptides were displaced by ammonia. Peptides have been shown to have a partition coefficient generally in favour of the phenol phase of a phenol–water mixture (Knight, 1951; Grassmann & Deffner, 1953). Countercurrent distribution with this system removed most of the dicarboxylic acids and partially concentrated the peptides relative to the free amino acids. The separation of the remaining free amino acids from the peptides was achieved by the technique described in a preceding paper (Carnegie, 1961). Peptides in acetic acid solution will migrate cationically, unless sulphonic or other strongly acid groups are present. The three-compartment cell removed uncharged substances, and also passage through the cellophan membrane into the cathode compartment ensured that substances collected in the cathode compartment had a molecular weight under 10 000. As the isolation of neutral peptides was the primary object of this investigation it was necessary, as a further precaution, to remove any acidic peptides, basic peptides and the free glutamic acid and aspartic acid which had been retained on the Dowex-1 (X8) resin and had migrated or diffused into the catholyte of the three-compartment cell. This separation was achieved by zone electrophoresis at pH 4. The mixture of supposed neutral peptides was then investigated with partition-chromatographic techniques.

Synge & Youngson (1961) have used L-[^{14}C]-valine to study the metabolic incorporation of this amino acid into a fraction similar to that studied in the present work and into fractions such as those previously studied by Synge & Wood (1958).

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MATERIALS AND METHODS

For the large-scale experiment described here, grass (approx. 6 in. long) was cut by scythe from a sward, grown from a seed mixture of 7 parts of Italian ryegrass to 10 parts of perennial ryegrass, on 21 May 1958. Three cwt. of sulphate of ammonia/acre had been applied in April and 2 cwt. of Nitrochalk (Imperial Chemical Industries Ltd.)/acre had been applied at the beginning of May. Grass (21·6 kg.) was subjected to the ether treatment of Synge (1951; cf. Chibnall, 1939) and the juice was expressed with a pressure of 2000 lb./in.^{2} The juice (volume 10·2 l.) was filtered through a pad of Celite 545 (Johns-Manville Co. Ltd., London). The general fractionation procedure is outlined in Fig. 1.

Chromatography on a cation-exchange resin (stage I)

This technique for preparing amino acids from plant extracts was first used by Westall (1950). The juice was processed in two equal batches. The filtered juice was slowly run (200 ml./hr.) through a column (12 cm. diam. × 17·5 cm. high) of Amberlite IR-120 A.G. resin (British Drug Houses Ltd., Poole, Dorset) in the H^+ form. After the juice was run in, the column was washed with water until the effluent was free from dry matter. A solution of NH_3 (sp.gr. 0·88, 175 ml./l.) was used as the displacing agent. The elution with NH_3 solution was continued until the effluent was shown, by evaporating and testing with ninhydrin, to be almost free from amino acids. The NH_3 eluate (6 l.) was concentrated by evaporation *in vacuo* at 35° to a small volume. A few drops of octan-2-ol (British Drug Houses Ltd.) were added to prevent frothing.

Countercurrent distribution in phenol–water (stage II)

Essentially the same procedure as described by Synge & Wood (1958) was used. The concentrated eluate (*SIB*) was not, however, acidified before shaking with phenol. Synge & Wood experienced little difficulty in separating the two phases, possibly because of the acidity, the presence of polyphenols or high concentration of inorganic salts. In this experiment the phases separated very slowly and it was necessary to centrifuge the contents of the funnels at 1300 rev./min. for 10 min. to obtain a separation. The first eight charges (250 ml. each) of phenol-rich phase were pooled and concentrated *in vacuo*.

Chromatography on an anion-exchange resin (stage III)

The technique described by Carnegie (1961) was used. Fraction *SIIB* (25·3 g. of dry matter, 3·2 g. of N) was brought to pH 8·6 with ammonia solution before it was transferred (125 ml.) to a column of Dowex-1 (X8) resin

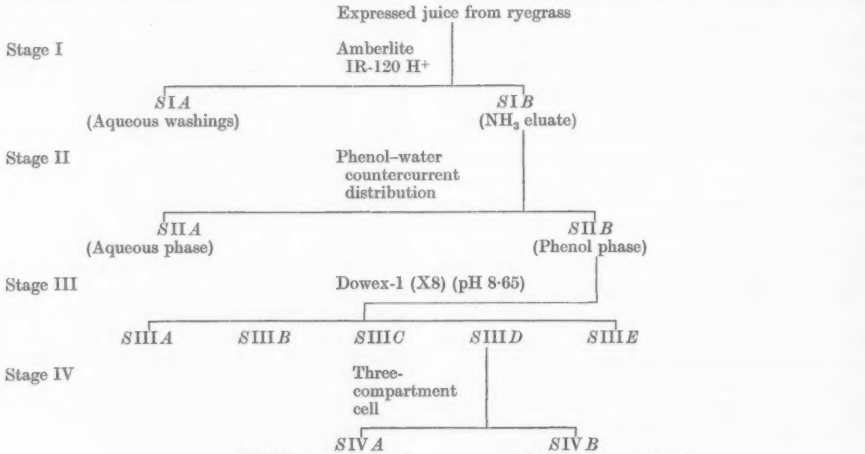

Fig. 1. Fractionation procedure (for details see text).

(13 cm. high × 10·5 cm. diam.). The chromatogram was then developed with 0·05M-buffer. The effluent *S*III*A* (1750 ml.) was dark brown in colour. The column was then washed with 2260 ml. of 0·2M-buffer to give eluate *S*III*B*. Displacement-development with 0·1N-HCl followed and the third fraction was collected. The HCl caused two pigment fronts to move down the column: the faster, dark brown in colour, was followed by a greyish-green zone. The slower front was reddish brown in colour. When approx. 1 l. had been collected in *S*III*C* the faster front was about 6 cm. from the foot of the column and the pH of the effluent became more acid. When 2 l. had been collected in *S*III*C* the 0·1N-HCl was replaced by N-HCl. A total volume of 2690 ml. of golden-brown eluate was collected in *S*III*C*. A precipitate slowly settled out in this fraction. Collection of *S*III*D* was started when the pigment associated with the Cl^- front was approx. 3 cm. from the bottom of the column. Just before the Cl^- front emerged the effluent became dark brown in colour. The volume collected in *S*III*D* was 1360 ml. Collection of *S*III*E* was started when Cl^- was first observed in the effluent and continued until all the dark-brown pigment was removed from the column (430 ml.). A pigment zone, which moved more slowly, was not collected. Samples were removed from the fractions for analysis. Fractions *S*III*A*, *S*III*B*, *S*III*C* and *S*III*D* were evaporated to dryness in a rotary evaporator *in vacuo* at under 40°. Fraction *S*III*E* was evaporated *in vacuo* at under 20° in a rotary evaporator whose receiving chamber contained 5 ml. of 5N-NaOH.

Ionophoresis in the three-compartment cell (stage IV)

The apparatus described by Synge (1951) was used with two cellophan membranes. Because of the tendency for dark-brown material to precipitate and block the cathode-compartment membrane, the middle-compartment solution was placed inside a small sac made from cellophan dialysis tubing, which held back the insoluble material; the sac was immersed in the same solvent in the middle compartment.

The cathode-compartment membrane was renewed several times during the course of a run. Portions (approx. 800 mg. each) of *S*III*C* and *S*III*D* were taken up in aqueous 5% (v/v) acetic acid and placed in the middle compartment (insoluble material in *S*III*D* was first removed by filtering). The anolyte was 0·1N-H_2SO_4 and the catholyte aqueous 1·0% (w/v) NH_3 solution. The catholyte was removed at 6-hr. intervals and replaced with 1·0% (w/v) NH_3 solution. The run was continued for 48 hr. The pooled catholytes were combined and evaporated to dryness *in vacuo* at under 40°.

Preparative paper electrophoresis at pH 4·0 *(stage V)*

A portion of *S*IV*B* (400 mg.) from *S*III*D* was taken through the fractionation procedure illustrated in Fig. 1 as far as stage VII. The quantities of material in the final fractions were too small for detailed study. The remaining catholytes were pooled and taken through the same procedure as described here.

The apparatus of Kunkel & Tiselius (1951), in which filter paper is pressed between two sheets of plate glass, was used. Because filter paper contains considerable quantities of bound amino acids (Wynn, 1949) it was necessary to wash the paper thoroughly before use. Whatman no. 17 paper (H. Reeve Angel and Co. Ltd., London) was washed by descending chromatography with 50% acetic acid for 24 hr., water for 12 hr., aqueous 0·25M-triethylamine (redistilled) for 24 hr. and finally with water for 24 hr. The paper was dried and two sheets (53 cm. × 19 cm.) were dipped into buffer, pH 4 (Grassmann, Hannig & Plöckl, 1955; pyridine 15 ml., acetic acid 50 ml., 2·5 l. of water), blotted and placed between two sheets of glass (43 cm. × 20 cm.), which had been smeared with silicone fluid M.S. 550 (Hopkin and Williams Ltd., London). The lower glass plate was clamped to a brass plate which was soldered to a cooling coil through which water at +2·5° flowed. The edges of the plates were sealed with polythene film smeared with silicone grease. The apparatus was set up and the paper left to equilibrate with buffer for 3 hr. before *S*IV*B* (400 mg.) was applied to a line 15 cm. from the anode end of the papers, approx. 2 ml. of buffer being used. A potential gradient of 11 v/cm. was applied for 6 hr. The pigment in *S*IV*B* moved slightly toward the cathode. At the completion of the run two prints were made on Whatman no. 1 paper by placing a sheet of no. 1 on top of the no. 17 paper and squeezing with a glass plate. The sheets were carefully removed, dried over a gentle heat, examined for fluorescence and the zones were marked. One of the prints was sprayed with ninhydrin and heated for 10 min. The other was treated by the chlorine–starch–KI method of Rydon & Smith (1952). From an examination of the prints and fluorescent areas zones were cut out for elution by descending chromatography, aqueous 50% (v/v) acetic acid being used in an apparatus similar to that of Dent (1947). Elution was continued for 12 hr., then the eluates were evaporated to dryness *in vacuo* below 40°.

Partition chromatography in butanol–acetic acid–water (stage VI)

*S*V*D* (166 mg.) was fractionated by chromatography in a column of kieselguhr with butanol–acetic acid–water (4:1:5, by vol.). Kieselguhr (Hyflo Supercel, Johns-Manville Co. Ltd., London) was washed with the phenol-rich phase of a phenol–water mixture until all the yellow colour was removed, then with water to remove the phenol. It was dried in the oven at 105° and spread on a tray to equilibrate with atmospheric moisture. Kieselguhr (22 g.) was mixed thoroughly with 14·7 ml. of aqueous phase of the freshly prepared solvent and made into a slurry with a few millilitres of the top phase and poured into a 2 cm. × 55 cm. column. The kieselguhr was carefully packed with a stainless-steel packing tool (Howard & Martin, 1950) to a height of 18 cm. The temperature was maintained throughout the run at 21·5±1°. *S*V*D* was quantitatively transferred to the top of the column with small volumes of top phase. Successive effluent fractions were collected, weighed and evaporated in tared flasks to dryness *in vacuo* below 40°. From an examination of the graph and from the appearance of the fractions it was decided which fractions to combine.

Preparative paper chromatography (stage VII)

The fractions obtained from *S*VI were fractionated with Whatman no. 3MM paper (washed with 50% acetic acid, then with water) and pyridine–*iso*amyl alcohol–water

(1:1:2, by vol.) (Edman, 1945). The material (approx. 20 mg./sheet) was transferred with a few μl. of water to a line 7 cm. from one end of a sheet of paper (53 cm. × 22·5 cm.) and dried with a hair-drier at room temperature. Descending development was carried out for 12 hr. at 21°. The papers were dried at a gentle heat and then examined for fluorescence. Strips 0·5 cm. wide were cut from the edges of the paper and treated first with ninhydrin then by the chlorine–starch–KI procedure. Zones were cut out according to the results of these examinations and eluted by descending chromatography with aqueous 10% (v/v) acetic acid. Paper blanks contained very little dry matter and no amino acids on hydrolysis. The fractions from *S* VI which contained more than 100 mg. were fractionated on Whatman no. 17 paper by ascending chromatography.

Preparative paper electrophoresis at pH 9·07 (*stage VIII*)

Only fraction *S* VIIA_4 was subjected to this procedure. Redistilled triethylamine (0·25 M) was converted into triethylamine carbonate with CO_2, 800 ml. of this solution was made to 2 l. and 400 ml. of 0·1 M-triethylamine added to give pH 9·07 (cf. Porath, 1956). Whatman no. 3MM paper (53 cm. × 19 cm.), washed as previously described for *S* V, was used in the apparatus of Kunkel & Tiselius (1951). *S* VIIA_4 (13 mg.) was transferred in a few μl. of buffer to a line 10 cm. from the cathode end of the paper and subjected to 6 v/cm. for 9 hr. The paper was removed, dried at a gentle heat and examined for fluorescence. A 0·5 cm.-wide strip was cut from one edge, sprayed with ninhydrin and heated for 10 min. Two zones, one staining yellow with ninhydrin and one staining purple, were cut out and eluted with 10% acetic acid.

Other analytical procedures

The methods were similar to those used by Synge & Wood (1958), with the following differences. For one-dimensional chromatography butanol–acetic acid–water (4:1:5, by vol.) and Whatman no. 1 paper were used. When paper-ionophoresis diagrams containing sodium tetraborate buffer were sprayed with ninhydrin solution, it was necessary to add about 1% of acetic acid to the spray. To detect copper complexes a 1% (w/v) solution of dithiooxamide (rubeanic acid) in aqueous 90% (v/v) acetone was used.

End-group analysis

The dinitrophenylation method described by Waley (1956) was used. For the chromatography of the *N*-2:4-dinitrophenyl (DNP) amino acids an improved solvent system was introduced: *tert.*-amyl alcohol (2-methylbutan-2-ol)–propan-2-ol (9:1, v/v) saturated with buffer, pH 7·15. Whatman no. 4 paper was saturated with this buffer made from 6·8 g. of KH_2PO_4, 10·65 g. of potassium hydrogen phthalate and 3·4 g. of NaOH/l. Sharp spots were obtained. The presence of phthalate ions probably reduced the tendency for the DNP amino acids to be adsorbed on to the paper with the production of tailing common with other solvent systems.

Quantitative amino acid analysis

A procedure similar to that of Moore, Spackman & Stein (1958) was used. The ninhydrin reagent was that of Connell, Dixon & Hanes (1955). For the basic amino acids a 20 cm. × 1 cm. column of Amberlite IR-120 resin (British Drug Houses Ltd.) was used.

RESULTS

Only the results relevant to the large-scale-fractionation procedure are given. The preliminary experiments on grass samples from previous years gave similar fractions, although the sequence of operations was not always the same as in the procedure described. Synge & Wood (1958) also observed that different batches of grass gave similar quantities of bound amino acids in their fractions.

From 21·6 kg. of spring grass (17·5% of dry matter, 4·0% of N in dry matter) 10·2 l. of juice was expressed. In Table 1 the values take into account the volume of juice which was not squeezed out of the grass and also allowance is made for any sample removed for analysis. When ammonia from the buffers contaminated the sample it was deter-

Table 1. *Results of fractionation of juice expressed from ryegrass*

Stage	Dry matter of fraction (% of dry matter of original grass)	N of fraction (% of N of juice)	N of fraction (% of dry matter of fraction)	Carboxyl nitrogen of fraction (% of N of fraction)	
				Before acid hydrolysis	After acid hydrolysis
Juice	29·0	100	2·3	40·0	60·0
S I *A*	21·5	25·0	0·8	20·0	37·0
S I *B*	3·5	59·0	11·3	52·0	77·0
S II *A*	2·4	38·0	13·1	60·9	79·1
S II *B*	1·1	20·0	12·6	22·8	55·8
S III *A*	0·66	12·2	12·2	36·9	53·7
S III *B*	0·07	1·1	11·3	16·3	53·6
S III *C*	0·05	1·3	16·1	9·3	25·9
S III *D*	0·12	1·6	9·2	21·4	69·8
S III *E*	0·07	0·9	8·8	9·5	119·6
S IV *B* from *S* III *C*	0·02	0·85	16·7	—	—
S IV *B* from *S* III *D*	0·04	0·37	8·2	15·0	40·0

mined by the Conway procedure and the nitrogen value corrected accordingly. The grass juice was of similar composition to that studied by Synge & Wood (1958). About 20% of the dry matter of the juice was not recovered from the Amberlite IR-120 resin. This material was probably composed of inorganic cations, organic bases stronger than ammonia and strongly adsorbed polyphenolic compounds. Both the ammonia eluate and aqueous washings were dark brown in colour. Since the chromatograms of samples from *SIA* failed to show any ninhydrin-positive substances it was probable that the CO_2 produced in the Van Slyke procedure was due to some artifact (cf. Synge, 1951). After hydrolysis a small amount of glutamic acid was present. Fraction *SIB* contained a complicated mixture of amino acids including γ-aminobutyric acid. The hydrolysate showed an increase in the proportion of the dicarboxylic acids and a spot with R_F (butanol–acetic acid–water; 4:1:5, by vol.) between those of valine and proline was no longer present. Most of the acidic bound amino acids studied by Synge & Wood would pass through the resin and be present in fraction *SIA*. The amount of dry matter in *SIB* was too great to be conveniently handled directly on the Dowex-1 (X8) column. Phenol–water countercurrent distribution concentrated certain peptides relative to the free amino acids and also removed most of the glutamic acid and aspartic acid. However, considerable quantities of the more acidic peptides, e.g. glutathione, would be retained in the aqueous phase. Several ninhydrin-positive spots on chromatograms of *SIIB* were no longer present after hydrolysis. In contrast *SIIA* showed little change in its amino acid pattern after hydrolysis.

The first fraction from the Dowex-1 (X8) column (*SIIIA*, 15 g. of dry matter) contained the free amino acids other than the dicarboxylic acids and a considerable amount of bound amino acids which were liberated on hydrolysis. An attempt was made to separate them from the free amino acids by precipitation with different concentrations of ethanol. Most of the brown pigment was precipitated by aqueous 85% (v/v) ethanol. This precipitate on paper electrophoresis at pH 9·2 migrated anionically slightly slower than glutamic acid and gave no colour with ninhydrin. On hydrolysis, it yielded a mixture of amino acids. It was possible that these bound amino acids were similar to the high-molecular-weight acidic compounds studied by Synge & Wood (1958). They were probably held by adsorption on the cation-exchange resin. They were probably too large to enter the particles of anion-exchange resin and exchange with the acetate ions. Fraction *SIIIB* (1·5 g.) was not studied in detail. Chromatograms before hydrolysis showed with ninhydrin two faintly purple spots, one near the leucine position and the other near the valine position. There was also a spot with R_F 0·42 in butanol–acetic acid–water which gave no ninhydrin colour but a strongly positive reaction with chlorine–starch–KI. Hydrolysates of *SIIIB* showed phenylalanine, leucine, valine, alanine, glycine, glutamic acid, aspartic acid and basic amino acids.

Fraction *SIIIC* contained material associated with the breakthrough of acetic acid from the column. It contained free glutamic acid and several ninhydrin-positive substances which were not present after hydrolysis. On a two-dimensional chromatogram (phenol–ammonia/collidine) one of these ran near leucine, one near valine and the other near glycine. Hydrolysates of this fraction contained a mixture of amino acids, the main ones being alanine, leucine, valine, glycine, glutamic acid and aspartic acid. Adenine as the free base was also a major component in this fraction. Its isolation is discussed below. Fraction *SIIID*, similar to *SIIIC*, was taken for detailed study. The fraction associated with the breakthrough of the Cl^- front, *SIIIE*, contained a faintly ninhydrin-positive substance which ran similarly to glutamic acid on a two-dimensional chromatogram. The hydrolysate of this fraction contained mainly glutamic acid and aspartic acid and only small amounts of the others. The high carboxyl-nitrogen value of the hydrolysates may be due to the aspartic acid content, since aspartic acid with ninhydrin liberates 2 mol.prop. of CO_2 (Van Slyke, Dillon, MacFadyen & Hamilton, 1941).

The results from the three-compartment-cell fractionation of *SIIID* and *SIIIC* are shown in Table 1. Separation of the cationic material was difficult because of the blocking of the cathode membrane by the high-molecular-weight pigmented material. A considerable proportion of the bound amino acids in fraction *SIIID* remained in the middle compartment with the brown pigment.

Table 2 shows the results of the fractionation of the cathode-compartment material *SIVB*. Free glutamic acid and aspartic acid were removed into fractions *SVB* and *SVC* from the main peptide fraction *SVD*, which remained close to the origin. The fractions near the cathode contained a higher proportion of lysine and arginine. Adenine, as the free base, was found in fractions *SVH* and *SVI*. In the preliminary experiments, with material equivalent to *SIVB* prepared from other batches of grass, adenine was isolated by paper electrophoresis at pH 9·0, with triethylamine carbonate buffer, and also by electrophoresis in 5% acetic acid. The isolated material was compared with adenine by paper chromatography in butanol–acetic acid–water (4:1:5, by vol.) and in propan-2-

ol–2N-HCl (65:33, v/v) (Wyatt, 1951). Its behaviour on paper electrophoresis at several pH values was also compared with that of adenine. It gave the same ultraviolet spectrophotometric curve as adenine. Its nitrogen content was 54·6% of the dry matter, compared with the theoretical 51·83% for adenine. In that experiment it accounted for 1% of the nitrogen of the expressed juice.

Several dinitrophenyl (DNP) amino acids were obtained on treating the *S*V fractions. DNP-aspartic acid and DNP-glutamic acid were observed in the anionic fractions. In the fraction *S*V*D* DNP-leucine and DNP-valine were the major products of dinitrophenylations with smaller proportions of DNP-glycine, DNP-alanine and DNP-glutamic acid. The yields of DNP amino acids and amino acids from the cationic fractions were much lower than would have been expected had all the dry matter of the fraction been composed of peptides.

The results of the fractionation of *S*V*D* on the butanol–acetic acid–water column are shown in Fig. 2. Chromatography of the pooled fractions in pyridine–*iso*amyl alcohol–water (1:1:2, by vol.) indicated that they were composed of a complex mixture of ninhydrin-positive substances and also substances which reacted strongly with chlorine–starch–KI. The relatively low yield of amino acids in the hydrolysates suggested that much of the dry matter of the fractions was not composed of bound amino acids. Fractions *E*–*H* gave only faint spots of amino acids and were not further investigated. Similar results were obtained on fractionating material from *S*III*C*.

Table 3 shows the results of preparative paper chromatography of *S*VI*A*. *S*VIIA_3 was highly crystalline and had a lemon-yellow fluorescence in ultraviolet light. No change in *S*VIIA_3 was observed after hydrolysis. The final yields of *S*VIIA_2 and *S*VIIA_4 obtained from pooling the products of both fractionations were 31 and 35 mg. respectively. The fractionation of *S*VI*B*, obtained from the second butanol–acetic acid–water column, is shown in Table 4. The yields of *S*VIIB_2 and *S*VIIB_3 were 37·6 and 67 mg. respectively. The major component of *S*V*D* was *S*VI*C*, which when fractionated by paper chromatography gave the results shown in Table 5. Most of the dry matter

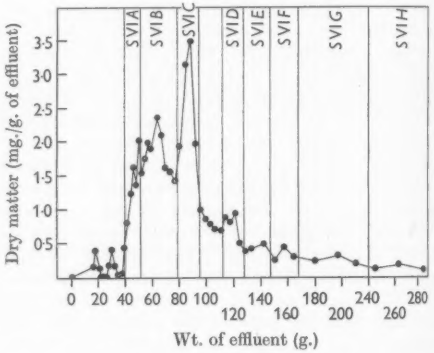

Fig. 2. Fractionation of *S*V*D* (166 mg.) by partition chromatography in butanol–acetic acid–water (4:1:5, by vol.). Column, 18 cm. × 2·0 cm. (22 g. of kieselguhr). Vertical lines indicate effluent fractions pooled for further study.

Table 2. *Results of preparative paper electrophoresis of S*IV*B from S*III*D*

Pyridine–acetic acid buffer, pH 4·0; Whatman no. 3MM paper; 400 mg. was applied; 11 v/cm.; 6 hr. For definitions of abbreviations used for amino acids (AA) see *Biochem. J.* (1957), **66**, 6.

Fraction	Distance from origin* (cm.)	Strength of colour with ninhydrin	Percentage of dry matter of *S*IV*B* recovered	Amino acids in acid hydrolysate	
				Major	Minor
*S*V*A*	+15·0–+10·0	+	1·8	Cys, Glu, Asp, Gly	Val, Leu, Phe
*S*V*B*	+10·0– +6·0	+ + +	5·0	Glu, Asp, Gly	Leu, Val, Ala
*S*V*C*	+6·0– +2·3	+ +	4·6	Gly, Glu, Ala, Val, Leu	Phe, Tyr, basic AA
*S*V*D*	+2·3– −3·0	+ + +	60·0†	Gly, Glu, Ala, Val, Leu	Phe, Thre, Ser, Tyr, basic AA
*S*V*E*	−3·0– −6·0	+ +	4·2	Gly, Glu, Ala, Leu	Phe, Thre, Ser, Tyr, Val, basic AA
*S*V*F*	−6·0– −9·5	−	1·8	—	Gly, Glu, Ala, Leu, basic AA
*S*V*G*	−9·5– −14·5	+	2·3	Gly	Basic AA, Ala, Val, Leu, Tyr
*S*V*H*	−14·5– −18·5	(Cl_2–starch–KI + +)	1·7	Gly	Basic AA, β-Ala, γ-Aminobutyric
*S*V*I*	−18·5– −21·0	+	0·6	Gly	—
*S*V*J*	−21·0– −24·0	−	0·2	—	—
*S*V*K*	−24·0– −27·0	+	0·6	Basic AA	Gly

* −, In direction of cathode; +, in direction of anode.
† Dry matter of *S*V*D* represented 0·025% of dry matter of grass.

Table 3. *Results of preparative paper chromatography of SVI A (0·003 % of dry matter of grass)*

Pyridine–*iso*amyl alcohol–water (1:1:2, by vol.); Whatman no. 3MM paper. See also protocol to Table 2.

Fraction	R_F	Colour		Dry matter of fraction ($10^3 \times$ % of dry matter of grass)	Major amino acids in acid hydrolysate
		Ninhydrin	Fluorescence		
$SVIIA_1$	0·087–0·13	Purple	White	0·2	Gly, Glu, Asp, Ala, Ser, Val, Leu, Phe
$SVIIA_2$	0·23 –0·45	Strong purple	Faint white	1·1	Same as $SVIIA_1$, but Phe stronger
$SVIIA_3$	0·50 –0·54	Bright yellow	Strong lemon yellow	0·24	Same as $SVIIA_2$, plus Tyr
$SVIIA_4$	0·55 –0·70	Purple	Faint	0·66	Leu, Phe, Val, Tyr

Table 4. *Results of preparative paper chromatography of SVI B (0·0083 % of dry matter of grass)*

Pyridine–*iso*amyl alcohol–water (1:1:2); Whatman no. 17 paper. See also protocol to Table 2.

Fraction	R_F	Colour	Dry matter of fraction ($10^3 \times$ % of dry matter of grass)	Major amino acids in acid hydrolysate
$SVIIB_1$	0·1 –0·25	Purple (ninhydrin)	0·32	—
$SVIIB_2$	0·33–0·44	Purple (ninhydrin)	2·7	Asp, Glu, Ser, Gly, Val
$SVIIB_3$	0·44–0·59	(Cl_2–starch–KI-positive)	5·4	Only Gly
$SVIIB_4$	0·65–0·71	Blue (fluorescence)	0·1	—

Table 5. *Results of preparative paper chromatography of SVI C (0·006 % of dry matter of grass)*

Pyridine–*iso*amyl alcohol–water (1:1:2, by vol.); Whatman no. 3MM paper. See also protocol to Table 2.

Fraction	R_F	Colour	Dry matter of fraction ($10^3 \times$ % of dry matter of grass)	Major amino acids in acid hydrolysate
$SVIIC_1$	0·0 –0·09	Faint purple (ninhydrin), brown pigment	0·5	Lys, Gly, Glu, Asp, Ala, Ser, Pro, Val, Leu, Phe
$SVIIC_2$	0·09–0·21	Strong purple (ninhydrin)	0·6	Same as $SVIIC_1$, plus Tyr
$SVIIC_3$	0·28–0·34	Strong purple (ninhydrin)	0·3	Same as $SVIIC_2$, less Pro
$SVIIC_4$	0·43–0·54	No ninhydrin colour; strong Cl_2–starch–KI reaction	3·4	Only faint Gly
$SVIIC_5$	0·64–0·76	Blue fluorescence	0·15	Traces of Ala, Leu, Val

was recovered in fraction $SVIIC_4$. The material in this fraction gave no colour with ninhydrin but a strong chlorine–starch–KI reaction. However, there were no amino acids in the hydrolysate other than a small amount of glycine. Also there was only about 2 % of nitrogen in the dry matter. The substance was not investigated further. Fraction *SVI D* obtained from the second column when fractionated by paper chromatography gave the results shown in Table 6. The yield of $SVIID_1$ was 10 mg.

Fractions $SVIIA_4$, $SVIIB_2$ and $SVIID_1$ were taken for further analysis. They gave single spots on a two-dimensional chromatogram when it was sprayed with ninhydrin (the pattern obtained is shown in Fig. 3). They were not crystalline and contained a greenish-brown pigment. Attempts to crystallize samples of these fractions with ethanol and butanol were not successful. Table 7 shows the results of quantitative amino acid analysis of these fractions with their approximate molar ratios calculated relative to aspartic acid. The separation of the small amounts of basic amino acids was not good. The modified ninhydrin reagent described by Connell *et al.* (1955) was used; their values for the extinction coefficients were used in the calculations. Only about 35 % of the dry matter of each of the hydrolysates was composed of amino acid residues yet nitrogen accounted for 12·4, 11·8 and 8·3 % of the dry matter of fractions $SVIIA_4$, $SVIIB_2$ and $SVIID_1$ respectively. Fractions $SVIIA_4$ and $SVIIB_2$ when dissolved in water both showed a rather flat maximum of ultraviolet absorption at 255–265 mμ with a small peak at 260 mμ ($E_{1\,cm.}^{0·001\,\%}$ 0·240).

It was obvious from these results that the

Table 6. *Results of preparative paper chromatography of SVI D (0·0025 % of dry matter of grass)*

Pyridine–*iso*amyl alcohol–water (1:1:2, by vol.); Whatman no. 3MM paper. See also protocol to Table 2.

Fraction	R_F	Colour with ninhydrin	Dry matter of fraction ($10^3 \times$ % of dry matter of grass)	Major amino acids in acid hydrolysate
S VII D_1	0·026–0·13	Pink	0·45	Asp, Glu, Ser, Thr, Gly, Ala, Val, Ileu, Leu, Phe, basic AA
S VII D_2	0·13 –0·26	Purple	0·29	Similar to *S* VII D_1
S VII D_3	0·26 –0·37	Yellow	0·1	Similar to *S* VII D_1
S VII D_4	0·49 –0·60	No ninhydrin (Cl_2–starch–KI reaction)	—	—

Table 7. *Approximate molar ratios of amino acids in acid hydrolysates of fractions relative to aspartic acid*

Amino acid	Fraction *S* VII B_2	*S* VII D_1	*S* VII A_4	*S* VIII A_1
Aspartic acid	1·00	1·00	1·00	1·00
Threonine	0·79	0·32	1·80	1·70
Serine	2·00	0·41	2·40	2·40
Glutamic acid	3·70	1·20	1·40	1·40
Proline	0·62	0·23	0·00	0·00
Glycine	0·35	0·97	12·00	2·50
Alanine	0·43	0·56	1·40	1·40
Valine	1·10	0·54	7·60	7·70
Methionine	Trace	Trace	0·00	0·00
Isoleucine	0·75	0·24	6·80	7·70
Leucine	1·40	0·34	22·00	20·70
Tyrosine	3·20	0·17	12·70	4·70
Phenylalanine		0·34	22·00	21·30
Lysine	0·30	0·19	Trace	Trace
Histidine	Trace	0·17	Trace	Trace
Arginine	Trace	0·19	Trace	Trace
Ammonia	7·70	1·00	4·00	7·30

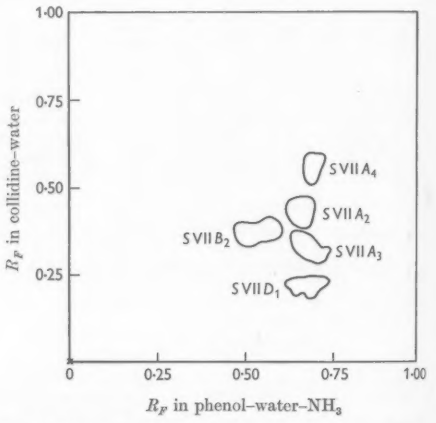

Fig. 3. Chromatography of fractions from stage VII in phenol–water–NH_3 (1 %, w/v) and collidine–water; Munktell OB paper; sprayed with ninhydrin.

fractions were still very complex, being composed possibly of a mixture of peptides and purine or pyrimidine type of compounds. Samples from *S* VII A_4, *S* VII B_2 and *S* VII D_1 were examined by paper electrophoresis in sodium tetraborate (pH 9·2) and in 5 % acetic acid. Spraying with ninhydrin revealed several purple-staining spots in all three, and treatment with chlorine–starch–KI showed some strongly positive spots which gave no reaction with ninhydrin. *S* VII A_4 showed in acetic acid a purple spot which had moved slightly further towards the cathode than leucyl-leucine and a bright-yellow-staining spot which had moved slightly less towards the cathode than glutamic acid. In sodium tetraborate the purple spot had moved slightly further than leucyl-leucine and the yellow spot had moved about half as far as leucyl-leucine. There was also a very strong chlorine–starch–KI-positive, ninhydrin-negative spot which had moved slightly less than the purple spot in sodium tetraborate. This substance was present in *S* VII B_2, which also contained several substances staining greyish purple with ninhydrin, with anionic mobilities in sodium tetraborate ranging from almost as fast as glutamic acid to only a slight anionic mobility. In acetic acid the bulk of the ninhydrin-positive material migrated cationically slightly faster than leucyl-leucine. The strongly chlorine–starch–KI-positive substance migrated cationically at the same rate as the main ninhydrin-positive material in acetic acid. Fraction *S* VII D_1 did not contain this chlorine–starch–KI-positive substance. It contained a mixture of ninhydrin-positive substances with the major proportion having an anionic mobility in sodium tetraborate approximately the same as that of glutamic acid. There was also some material with an anionic mobility slightly greater than that of glutamic acid. In acetic acid the major component had a cationic mobility between those of glutamic acid and leucyl-leucine.

The material in SVIIA_4, which gave a purple colour with ninhydrin, was isolated by preparative paper electrophoresis with triethylamine carbonate buffer, pH 9·07. This material SVIIIA_1 represented approx. 0·00015% of the dry matter in the grass. Most of the dry matter of fraction SVIIA_4 was recovered from the chlorine–starch–KI-positive zone. The molar ratios of the amino acids in a hydrolysate of SVIIIA_1 are shown in Table 7. SVIIIA_1 is similar to SVIIA_4 except that SVIIIA_1 contains a lower proportion of glycine and tyrosine.

DISCUSSION

A detailed study of the nature of the peptide-like compounds in the final fractions was not possible because of the lack of material. Further work will be required to isolate substances of sufficient purity for a detailed study of their structure to be made. However, the material in several of the fractions isolated was similar to a mixture of oligopeptides. For instance, fraction SVIIIA_1 appeared to contain a mixture of peptides similar to peptides of leucine. They had similar R_F values to those of leucyl-leucine in three different solvents and behaved similarly on electrophoresis at two different pH values. When the SVI fractions were treated with copper phosphate and then subjected to paper electrophoresis according to the procedure of Carnegie & Synge (1961), complexes were formed which migrated anionically as streaks. However, no colour was observed (possibly because traces of pigment masked it) and the yield of complex was low because of the non-peptide material present in the fractions. Pardee (1951) suggested that if the qualitative amino acid composition is determined for a peptide of known R_F then the approximate number of amino acids in the molecule can be calculated. If the major peptides present in SVIIIA_1 are assumed, from the amino acid analysis, to be composed of leucine, isoleucine, phenylalanine, valine and tyrosine, then with the formulae of Pardee and the determined R_F, the approximate number of amino acid residues per molecule can be calculated. The value obtained for n, the number of residues, from the R_F in pyridine–*iso*amyl alcohol–water was 2·5, and in phenol–water–ammonia was 4·5. DNP amino acid analysis on fractions from which SVIIIA_1 was derived indicated that leucine was one of the major *N*-terminal amino acids. The properties of this group of compounds are all similar to those expected for a mixture of small peptides such as leucyl-leucine, leucylvaline, isoleucyl-leucine etc. However, all the dry matter in this fraction was not accounted for by amino acid residues. It is possibly contaminated by some of the strongly chlorine–starch–potassium iodide-positive material, which had a slightly lower mobility at pH 9. The absorption in ultraviolet light at 260 mμ by fractions SVIIA_4 and SVIIB_2 would suggest the presence of purine or pyrimidine types of compound. These would also account for the non-amino acid nitrogen present in these fractions, which, when hydrolysed, were shown to contain only about 35% of amino acids in the dry matter. Ferguson & Terry (1953, 1954) found that approximately 7% of the non-protein nitrogen of grass was composed of purine-type compounds. Their identification of free adenine in grass (Ferguson & Terry, 1953) is confirmed.

Further work will be required to ascertain if these purines are merely contaminants of the peptide fractions or if they are in fact bound chemically to the peptides. This is the more important because of several recent reports of the isolation, with techniques similar to those used in the present work, of 'peptide-nucleotides' from plant material. Thus Hase, Mihara, Otsuka & Tamiya (1959) (cf. Hase, 1958) extracted water-soluble fractions from *Chlorella* and *Saccharomyces* similar in amino acid composition to those described here. Davies & Harris (1960) (cf. Harris, Davies & Parsons, 1958) have isolated similar substances from yeast, one of which they consider to be arginylalanylarginylalanyl-5′-uridylate. Gilbert & Yemm (1958) have made similar observations on *Torulopsis*. Medvedev & Zabolotskii (1958) found 2–3% of a similar group of amino acids bound in ribonucleic acid isolated from higher plants.

In further investigation of the possible occurrence of peptide–nucleotides it would be desirable to develop methods that subject the material being fractionated to less-extreme pH values than those used here, since the possibility of breakdown of such compounds in the course of the fractionation has by no means been excluded. The further possibility that these peptide-like substances form a complex in some way with perhaps carbohydrate or lipid moieties cannot be ruled out until they have been more highly purified.

Fractions SVIIB_2 and SVIID_1 differed from SVIIA_4 in their higher proportion of aspartic acid and glutamic acid. Their properties likewise were consistent with those of oligopeptides.

Since the methods used were designed to recover, as quantitatively as possible, neutral oligopeptides, then it can be estimated that they account for less than 1% of the nitrogen of the expressed juice. Synge & Wood (1958) obtained similar results with a different fractionation procedure. It was possible that some of these peptides were produced by autolysis of protein during the time the grass was standing in ether. However, similar 'peptide' fractions were isolated from extracts prepared by plunging grass into boiling water immediately after cutting. Also in experiments by Synge & Youngson

(1961), with [^{14}C]valine, a similar 'peptide' fraction was obtained when either the ether procedure or extraction with boiling water was used. The yield of bound amino acids in the fraction from the ether procedure was, however, higher than in that from the boiling-water extraction. No single substance was found to be present in a much higher concentration than the others. The material isolated seems to be composed of a mixture of closely related substances differing slightly in their amino acid composition.

Synge & Wood (1958) observed that only a small proportion of the bound amino acids could be genuine oligopeptides, the greater part being in acidic compounds devoid of ionizing basic groups. The experiments described here with a different fractionation procedure confirm their observation. Several of the more acidic bound amino acids which were associated with dark-brown pigment were adsorbed on the cation-exchange resin and displaced during the ammonia development. Their presence in the supposedly cationic fraction should serve as a warning in assessing the results obtained when ion-exchange resins are used to isolate compounds from biological materials. The adsorption of polyphenolic compounds from plants on a cation-exchange resin and their elution with ammonia was observed by Thompson, Morris & Gering (1959).

Other reports of peptide-like substances in plants

This topic has been reviewed (Bricas & Fromageot, 1953; Synge, 1955, 1959; Synge & Wood, 1958). Apart from instances dealt with by these authors and the 'peptide–nucleotide' substances mentioned above, the following better-defined reports may be added. γ-Glutamylvalylglutamic acid was isolated from *Juncus conglomeratus* by Virtanen & Ettala (1958). Montant (1956) mentioned a fraction from the latex of *Euphorbia characias*, which yielded on hydrolysis glutamic acid, leucine, valine and glycine. Rinderknecht, Thomas & Aslin (1958) (cf. Zacharius, Morris & Thompson, 1959) isolated γ-glutamyl-*S*-methylcysteine from extracts of beans. Two other peptides, γ-glutamyl-*S*-methylcysteine sulphoxide and γ-glutamyl-leucine, were tentatively identified. Peptides of arginine, citrulline, glycine, threonine and ornithine have been isolated from Irish moss (Young & Smith, 1958; cf. Morita, 1955). Ohira, Imai & Kido (1955), Ohira, Kido & Imai (1955) and Rudinger & Pravda (1958) have reported work bearing on the structure of eisenin and related seaweed peptides. Makisumi (1958) isolated arginylglutamine from *Cladophora* sp. Butler & Bathurst (1958) recognized rather large quantities of bound γ-aminobutyric acid in root nodules of *Trifolium* spp. A toxic peptide isolated from a blue–green alga *Microcystis aeruginosa* was found to contain L-aspartic acid, L-glutamic acid, D-serine, L-valine, L-ornithine, L-alanine and L-leucine, probably in a cyclic structure (Bishop, Anet & Gorham, 1959). In further purification of viscotoxin Samuelsson (1958, 1959) has eliminated carbohydrate claimed to be present by earlier workers and obtained a substance diffusible through cellophan and yielding 11 different amino acids after hydrolysis (see also Porath, 1960). Kauffmann & Ködding (1957) found bound glycine, alanine, serine, aspartic acid and glutamic acid in lipid fractions from spinach leaves and Kauffmann (1957) found the same amino acids 'loosely bound' to grana-protein of various plants. Reindel & Bienenfeld (1956) found, in virus-infected potato leaves, a peptide composed of glycine, aspartic acid, glutamic acid, threonine, alanine, valine, leucine, a little methionine and some phosphorus. Fowden (1958) has established that the acetylornithine found in plants is the N^{δ}-derivative, and has found it in a number of grasses. Guha & Das (1957) (cf. Das & Ghosh, 1957; Das & Guha, 1957, 1958) isolated from bran a crystalline substance, mainly peptide in character, containing bound nicotinic acid. Two peptide-like compounds have been observed in corn sap by Fejér & Kónya (1959), who give references to earlier similar observations. Bollard (1957) observed the presence of peptides in apple tracheal sap. Sehon & Richter (1959) isolated an allergenically active peptide-like fraction from ragweed pollen. These last three groups were not well characterized chemically. Miettinen (1959), in an investigation of the incorporation of DL-[^{14}C]-alanine by pea roots, isolated a peptide composed of equimolar amounts of alanine and glutamic acid with less glycine and serine and traces of threonine, valine and leucine, a mixture rather similar to that from several of the fractions discussed here.

SUMMARY

1. A complex mixture of neutral amphoteric peptide-like compounds of low molecular weight has been isolated from the expressed juice of ryegrass. The bound amino acid residues account for less than 1 % of the nitrogen of the expressed juice.

2. The techniques used in the isolation included ion-exchange chromatography, partition chromatography and preparative paper electrophoresis. Pigments were difficult to remove. Nitrogenous bases similar in some of their properties to purines and pyrimidines occurred in several of the fractions.

3. A group of substances with amino acid content mainly of leucine, isoleucine, valine, tyrosine and phenylalanine was isolated. They had similar chromatographic and electrophoretic properties to leucyl peptides of low molecular weight. The

presence of other groups of substances, containing a higher proportion of glutamic acid and aspartic acid and smaller amounts of most of the common amino acids, was observed. It was not possible, because of the small quantities available, to make a full characterization.

4. The occurrence of adenine as a free base in grass is confirmed.

I am grateful to Dr R. L. M. Synge for generous advice and encouragement. My thanks are also due to other colleagues at the Rowett Research Institute, especially Dr J. L. Bailey, for advice, help and equipment, and to Miss C. Blair for technical assistance with chromatograms. I am grateful to the Agricultural Research Council for a Studentship. This work forms part of a PhD. Thesis to be submitted to the University of Aberdeen.

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Metabolic Incorporation of L-[^{14}C]Valine into Protein and Bound Non-Protein Forms in Italian Ryegrass

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(*Received* 3 *May* 1960)

Aqueous protein-free extracts of Italian ryegrass contain a complicated mixture of substances which on acid hydrolysis yield amino acids. About 5% of the nitrogen of these extracts is contributed by such bound amino acid residues. Compounds of this kind, apparently devoid of basic ionizing groups, have been studied by Synge & Wood (1958); Carnegie (1961*b*) has studied those possessing basic groups. No individual compounds, apart from pyrrolidone carboxylic acid, which is probably an artifact, have yet been identified in either of these classes.

It should be possible, after administering an isotopically labelled amino acid to the plant and measuring incorporation of its residues into compounds of these two classes as well as into protein, to obtain a view of the metabolic relationships of these non-protein compounds of amino acids. We were particularly concerned whether they could be intermediates in the anabolism or catabolism of protein. We used generally-labelled L-[^{14}C]valine because valine residues are present in reasonable abundance in these non-protein compounds and because valine is not metabolized into other amino acids to any appreciable extent; this is the general experience in work with animals (e.g. Campbell & Work, 1952; Harris & Watts, 1958) and seems also to be true for plants. Miettinen (1959) has described a slight conversion (1–2%) of DL-[^{14}C]-valine administered to pea plants through the roots into γ-aminobutyric acid and leucine–isoleucine in the course of 3 hr. It is also technically simple to isolate valine as its *N*-2:4-dinitrophenyl derivative for determination of its amount and specific activity.

We wished the physiological conditions to be close to those of active photosynthesis and growth. For this, injection into the stem of the intact growing plant [McConnell and colleagues, see McConnell (1959) and earlier papers in this series] or uptake through the root (Miettinen, 1959) seem the best means of introducing labelled non-volatile compounds. However, the former method might complicate the recovery of labelled material, if any had entered the root. Moreover, the previous chemical studies on which we based our analytical procedures had related only to the aerial parts of the plant.

Accordingly we severed the stalk near the ground and introduced amino acid of high specific activity in water by the transpiration stream across the cut surface (cf. McCalla & Neish, 1959). Metabolism was allowed to proceed in the open air on fairly bright days for rather short periods. We hoped that under these conditions there would be some net synthesis of protein and that the breakdown which occurs when severed leaves are kept in water for longer periods would be avoided (for a review see McKee, 1958). Although we did not measure changes in total protein, we found no evidence for net catabolism of protein, in that none of the fractions of non-protein nitrogen, expressed as a percentage of the total nitrogen of the plant, increased during our experiments above the values found on analysis of freshly severed material. Stephenson, Thimann & Zamecnik (1956) incubated tobacco-leaf disks over a liquid medium containing L-[^{14}C]valine and observed vigorous incorporation of ^{14}C into the protein, presumably as valine residues. Incorporation was stimulated by light. Arnstein & Clubb (1957), who studied the incorporation of [^{14}C:^{15}N]valine into mycelial protein during growth of *Penicillium chrysogenum*, observed good incorporation of the carbon skeleton, with substantial transfer of ^{15}N to other amino acids, etc., by transamination or deamination.

MATERIALS

L-[$^{14}C_5$]*Valine*. This was purchased from The Radiochemical Centre, Amersham, Bucks. (CFB 21, batch 8, specific activity 68·5 μc/mg.). Six months after its analysis at Amersham, during which time it had been stored as received (in a sealed ampoule in the dark at room temperature), 0·1 mc was accurately subdivided into portions bearing a known ratio to one another by evaporation of weighed portions (approx. 1 μc and 10 μc) of an aqueous solution in neutral-glass ampoules, which were sealed under a vacuum of 0·1 mm. Hg (after drying overnight over P_2O_5 at this pressure) and stored at room temperature in the dark until required. Two-dimensional filter-paper chromatograms showed, on radioautography (see below), besides the main valine spot a subsidiary radioactive spot having R_{Val} 0·93 in phenol–NH_3 and R_{Val} 1·41 in 'collidine', which we judged to have about 2% of the activity of the main spot. This component was not found on radioautographs of the diffusates or of hydrolysates of protein fractions from grass into which [^{14}C]valine residues had been

incorporated. Tolbert *et al.* (1953) (cf. Lemmon, 1953) observed 1–2% of a non-volatile product of radiochemical decomposition on storage of [$\gamma\gamma'$-^{14}C]valine (6·0 μc/mg.) for 15 months. The experiments described here were completed within 1 year of the analytical checking of the material at The Radiochemical Centre.

N-2:4-*Dinitrophenyl*-L-*valine.* DL-Valine (Marvel & du Vigneaud, 1943; Marvel, 1955) was resolved to L-valine according to Fischer (1906). This gave a single spot on filter-paper chromatograms (as below); $[\alpha]_D^{20}$ + 28·6° (6 N-HCl, *c*, 4). From this, working always in subdued artificial light, the dinitrophenyl (DNP) derivative was prepared according to Rao & Sober (1954). When first prepared, yellow crystals were obtained, which softened at 129° before melting at 131° (uncorr.); $[\alpha]_D^{20}$ – 39° (acetic acid, *c*, 1), with slow positive mutarotation. On all later occasions redder crystals were obtained, which melted sharply at 131° and gave $[\alpha]_D^{20}$ – 28° (no mutarotation). Rao & Sober (1954) reported m.p. 132°; $[\alpha]_D^{24-26}$ – 28°. Campbell & Work (1952) reported m.p. 129°. It seems possible that the crystals first obtained were of some metastable tautomeric form of the compound.

DNP-L-[^{14}C]valine for use as a standard in comparisons of specific radioactivity was prepared as described above from L-[^{14}C]valine diluted with a known weight of inactive L-valine.

METHODS

General. Unless otherwise stated, evaporations were done *in vacuo* below 40°. Optical rotations were determined in a 0·5 dm. tube. Dry matter, N and carboxyl N (ninhydrin CO_2) were determined on the non-radioactive controls as described by Synge & Wood (1958). Two-dimensional paper chromatograms were also made by their procedure.

Results are reported on the basis of total dry matter or total N of the original grass, after making appropriate allowance for subdivisions, removal of samples etc.

Outline of fractionation procedures. These were based on the work of Synge & Wood (1958) and Carnegie (1961*a*, *b*) and are shown in Fig. 1. The different categories of bound amino acids were isolated largely in fractions 5 and 8.

Cutting of grass and administration of L-[^{14}C]*valine.* On a fairly bright and breezy day three stems of Italian ryegrass, growing on the Duthie Experimental Stock Farm, were cut just above a node and the bottom leaf was stripped back from the stem. The stems (wet wt. about 4 g.) were placed in an opened ampoule containing [^{14}C]valine (1 μc) dissolved in 100 μl. of water. The stems imbibed this solution in a few minutes. Further portions (50 μl.) of water were then added as soon as each portion was nearly sucked up. This was done in the field for the time stated, after which the grass and tube were quickly taken to the laboratory. Control stems cut at the same time were supplied with water only.

Extraction and washing. The grass and tube were immediately placed in a large beaker covered with a clock glass and containing 200 ml. of briskly boiling water. After 10 min. boiling the beaker and contents were quickly cooled to room temperature and the aqueous extract was poured off the leaf residues. The residues were brought to the boil with two further portions of 200 ml. of water and these washings were added to the initial extract. The residues were further washed in the same way at the boil with five 100 ml. portions of 0·05% commercial L-valine in aqueous 1·2% (v/v) acetic acid, followed by two 100 ml. portions of water. These washings were discarded. The residues were spread out and dried between filter papers for radioautography (fraction 1).

In expt. IV the grass was extracted thrice with water in the presence of excess of ether at room temperature (in all about 30 min.). The residues were then washed with boiling solutions as described above. With the non-radioactive controls, valine was omitted from the wash liquor and the washings were collected for analysis (fraction 2).

Dialysis. The extract was evaporated to small volume and dialysed in cellophan against approx. 500 ml. of water saturated with chloroform for several days at 0–1°, with stirring at intervals. After measuring volumes of diffusate (fraction 3) and bag contents, the latter were discarded (in the non-radioactive experiments their N and dry-matter were determined, so that total initial N and dry matter of the grass could be calculated). In calculating results on fractions derived from the diffusate, it was assumed that its components had been present in equal concentration in the bag contents.

Countercurrent distribution in phenol–water. The diffusate was evaporated to small volume, adjusted to pH 2 by addition of 2 N-H_2SO_4 and transferred with water (final vol. 20 ml.) to the first of a train of five separating funnels. We here followed the procedure of Synge & Wood (1958), 20 ml. charges of each phase being used; the first eight charges of phenol-rich phase to leave the train were pooled and evaporated to small volume with water as described by them (fraction 4). The residues in the train were discarded.

Ionophoresis in three-compartment diaphragm cell. Fraction 4 was transferred in about 15 ml. of aqueous 10% (v/v) acetic acid to the middle compartment of the cell (Synge, 1951; Synge & Wood, 1958). The catholyte was aqueous 1% (w/v) NH_3 and the anolyte was 0·1 N-H_2SO_4. After 6 hr. running the catholyte was removed and its compartment was recharged with fresh 1% NH_3 solution. After 24 hr. the middle-compartment contents were collected and evaporated to dryness (fraction 5).

The 6 hr. catholyte was evaporated to dryness and run again for 6 hr. in a fresh cell as described above. The final catholyte (fraction 6) was collected and evaporated to dryness, finally in a high vacuum over H_2SO_4 and soda lime at room temperature to remove the bulk of the ammonium acetate present.

Fractionation on Dowex 1-X4. The product was chromatographed in ammonium acetate buffer (pH 8·69) on a column (1 cm. × 6 cm.) of Dowex 1-X4 by elution-development with 0·05 M-buffer as described by Carnegie (1961*a*, *b*). After 25 ml. of effluent had been collected (fraction 7), the column was developed with N-HCl and effluent was collected until breakthrough of Cl^- (fraction 8). These fractions were evaporated as described above to minimize the content of ammonium acetate.

Acid hydrolysis. Fractions 1 (after radioautography), 5 and 8 were hydrolysed by refluxing for 24 hr. in excess of 6 N-HCl. The hydrolysates were evaporated to dryness *in vacuo* up to 100°.

Isolation and determination of valine as its dinitrophenyl derivative. This was done on suitable portions of hydrolysates of fractions 1, 5 and 8 and of fractions 7 (unhydrolysed). The general procedure of Perrone (1951) and Krol (1952) was followed (cf. Campbell & Work, 1952; Muir,

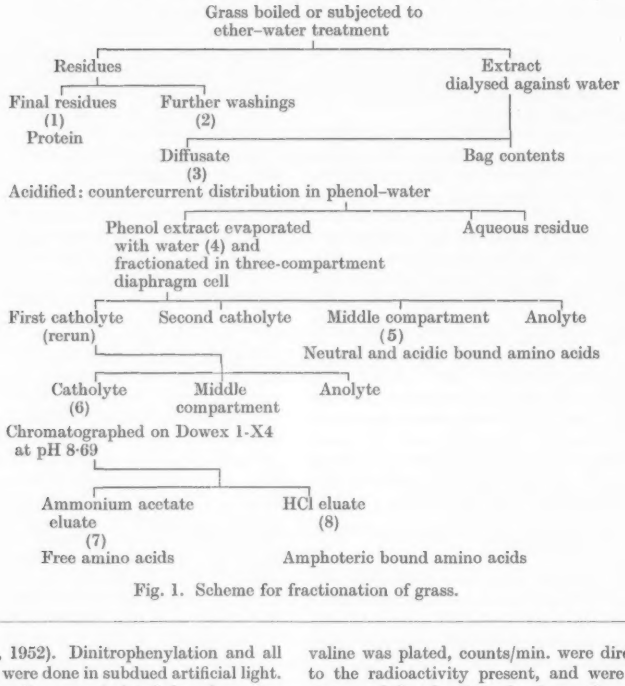

Grass boiled or subjected to ether–water treatment
Residues
Extract dialysed against water
Final residues (1) Protein
Further washings (2)
Diffusate (3)
Bag contents
Acidified: countercurrent distribution in phenol–water
Phenol extract evaporated with water (4) and fractionated in three-compartment diaphragm cell
Aqueous residue
First catholyte (rerun)
Second catholyte
Middle compartment (5) Neutral and acidic bound amino acids
Anolyte
Catholyte (6)
Middle compartment
Anolyte
Chromatographed on Dowex 1-X4 at pH 8·69
Ammonium acetate eluate (7) Free amino acids
HCl eluate (8) Amphoteric bound amino acids

Fig. 1. Scheme for fractionation of grass.

Neuberger & Perrone, 1952). Dinitrophenylation and all subsequent operations were done in subdued artificial light. Dinitrophenyl (DNP)-valine was isolated by chromatography with ether on a column of Hyflo-Supercel (Johns-Manville Co. Ltd., London) charged with 0·25 M-sodium phosphate buffer, pH 6·7, followed by rechromatography on a similar column in 2% (v/v) butanol in chloroform. The effluent (30 ml.) was extracted with 10 ml. of 0·05 M-sodium phosphate buffer, pH 11·3, and E at 410 mμ was determined on this solution in a Unicam SP. 1400 spectrophotometer with a 1 cm. cell, reading against a blank of the same solvent. DNP-L-valine under these conditions gave a linear calibration curve, corresponding to E 0·274 for 100 μg. Control recoveries of valine from a made-up mixture of amino acids simulating a leaf-protein hydrolysate averaged 110%, probably due to contamination by other DNP derivatives. No correction has been made for this.

Determination of radioactivity. All counting was done on DNP-valine samples under 'infinite thinness' conditions. The aqueous phase (recovered quantitatively with water from the spectrophotometer cell) was acidified with HCl and swirled in a separating funnel with ether (5 ml.). The ether phase was washed thrice with 15 ml. portions of 0·1 N-HCl and evaporated to dryness. The residue was transferred quantitatively in acetone to an aluminium planchet (AERE Harwell Drg. no. A. 36688; 2·25 cm. diam.) on which a circle of 105 lens tissue (J. Green, Maidstone) had been fixed by dropping on to it 3 drops of a 0·25% (w/v) solution of Perspex in chloroform. When dry, the sample was counted with a Geiger–Müller end-window counter. Corrections were made for background and coincidence. Provided that less than 100 μg. of DNP-valine was plated, counts/min. were directly proportional to the radioactivity present, and were unchanged after storage of the planchets for 6 months at room temperature. Counting efficiency was approx. 5%. For calculating the recoveries in Table 3, reference was to the standard DNP-L-[^{14}C]valine spread for counting in the same manner.

Radioautography. Paper chromatograms were dried at 105° for 1½ hr. before radioautography. The chromatogram or pressed-grass specimen was opposed to Ilfex safety-base X-ray film (Ilford Ltd., Ilford, Essex); after a suitable exposure the film was developed as recommended for X-ray work.

RESULTS

Control expt. (C) and corrections used in calculations. Instead of the introduction of [^{14}C]valine under conditions favouring its metabolism, freshly cut grass was placed immediately in boiling water containing the [^{14}C]valine. On preparation, no radioactivity was detected in the protein valine (i.e. $< 0{\cdot}1$% of the total radioactivity initially added). This grass was cut on 8 April 1958 at 10.00 hr. and consisted entirely of short tender leaves.

The free valine present in this grass specimen was calculated by isotope dilution to be 0·13% of the total N of the grass specimen (Table 2). On this basis, 32% of the free valine was recovered in fraction 7. Most of the loss was presumably into the aqueous residue from the countercurrent distribution. In expts. I–IV the percentages of free

valine and of radioactivity in free valine (Tables 2 and 3) have been calculated from results on fraction 7, assuming the same recovery.

If the radioactivity of valine in the hydrolysates of fractions 5 and 8 in expt. C was assumed to be due to contamination of these fractions by the free valine present in the cell catholyte, put on to the Dowex 1-X4 column and recovered in fraction 7, the contaminations amounted respectively to 0·046 and 0·30 % of this free valine. In calculating the results for expts. I–IV, corresponding percentages of the radioactivity of valine in fraction 7 have been subtracted from the radioactivity of the valine in hydrolysates of fractions 5 and 8; the amounts subtracted in no case exceeded 30 % of the radioactivity found.

Expts. I–IV. Expts. I (1 hr. transpiration) and II (2 hr. transpiration) were done on 4 June 1958 between 10.30 and 12.30 hr. (temp. 16–18°). The grass was just about to flower (Fig. 2*a*), and was selected from a well-fertilized patch where growth and depth of green were above average.

Expts. III and IV (2 hr. transpiration) were done on 12 August 1958 between the same hours (temp. 19–23°). The grass was at the flowering stage (Fig. 2*b*). As before, the tallest and greenest grass was selected. Stems were assigned to each ampoule in pairs whose members resembled each other as closely as possible; III was then prepared with boiling water as usual and IV by the ether–water procedure.

Incorporation of valine into grass protein. Radioautography of the boiled-out residues (fraction 1) showed that in grass not yet flowering the radioactivity was mainly fixed in the lower part of the stem near the point of entry (Fig. 2*a*), whereas at the flowering stage there was widespread fixation mainly in leaves and flower, with much less in the stem (Fig. 2*b*).

The two-dimensional chromatograms of hydrolysates of fraction 1 showed a single radioactive spot in the valine position. A partial hydrolysate (10N-HCl for 96 hr. at 37°) showed numerous faint radioactive spots, a majority of which ran fast in the phenol phase. These observations, taken with our inability to detect radioactivity in residues (fraction 1) of leaves boiled with [^{14}C]valine (expt. C) leave little doubt that the [^{14}C]valine was incorporated into protein by a metabolic process.

Analytical results on grass specimens. These are given in Tables 1 and 2. The high protein content of C, as well as the high proportion of non-protein to total nitrogen, reflect the immaturity of the specimen.

Distribution of radioactivity in grass fractions. Table 3 shows the recoveries (expressed as percentages of the radioactivity initially administered) of radioactive valine from the different fractions studied, and also total recovery of radioactivity in the form of valine residues. The amount by which this falls short of 100 % gives an idea of the extent of metabolism of the valine administered into other chemical compounds during each experiment.

In Table 4 the specific radioactivity of the valine isolated from each fraction is expressed as a percentage of the specific activity of the free valine present in the grass at the end of the experiment.

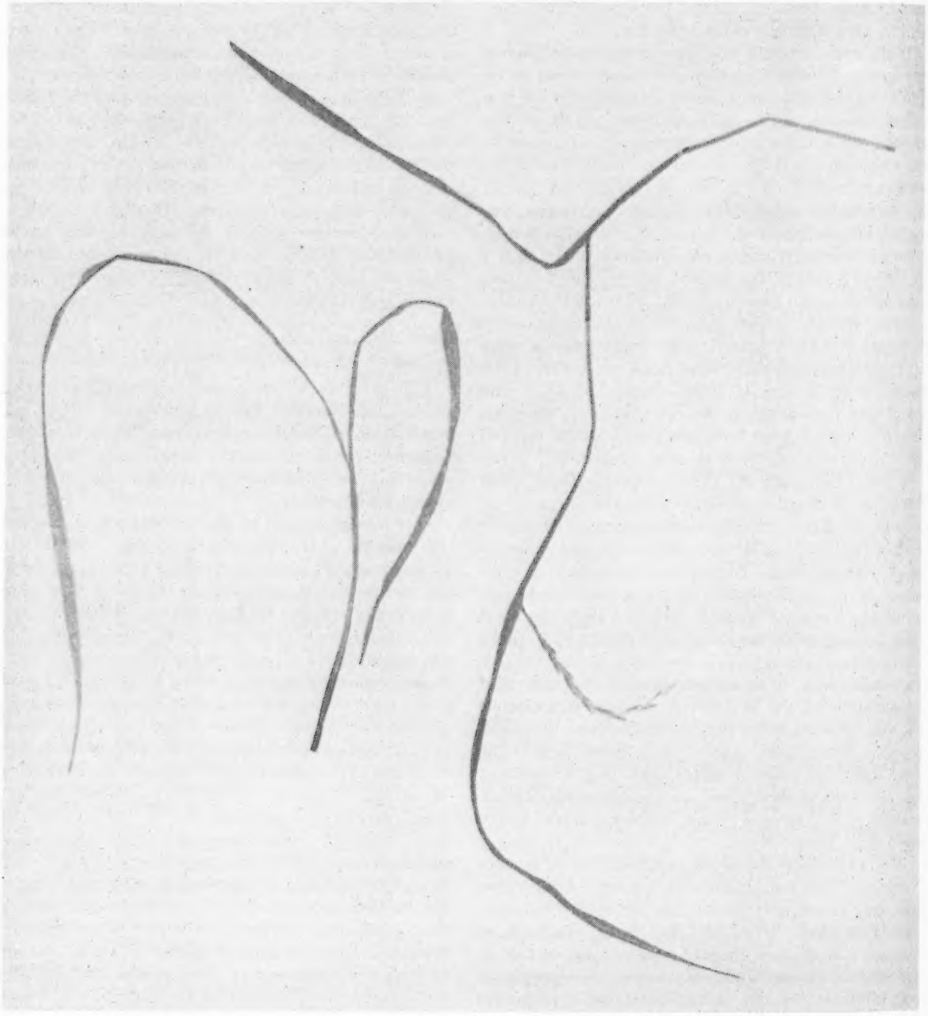
Radioautography of two-dimensional chromatograms of diffusates in expts. III and IV showed only free valine, with a diffusely blurred background, presumably due to the spreading of the small amount of other radioactivity over a large number of components.

DISCUSSION

It is clear that substantial incorporation of free valine into protein was taking place under the conditions of these experiments. This is a prerequisite for a metabolic experiment aimed at searching for possible intermediates in the biosynthesis of protein.

Our interpretation of the present results is that the specific activities of the bound non-protein valine in acidic and neutral compounds (fractions 5) and in amphoteric compounds (fractions 8) are not sufficiently close to that of the free valine for any substantial proportion of such bound valine to have been serving as an intermediate stage in the incorporation of free valine into protein. We regard it as specially significant that the amounts and specific activities of the valine in these non-protein compounds were highest in expt. I, which of all the experiments shows the least incorporation of valine into protein. In this experiment, also, the total recovery of radioactivity in valine residues was smallest, which suggests that the entire metabolism of valine was for some reason diverted away from protein synthesis. These compounds, on the other hand, had specific activities too high to arise by indiscriminate catabolism of pre-existing proteins. It seems most reasonable to regard them as being metabolites of free valine unconnected with protein synthesis. The amphoteric fractions 8 in each experiment showed higher specific activity than the neutral and acidic fractions 5, and may therefore possibly have been precursors of them.

It remains a possibility that some minor components of fractions 5 or 8 contained valine residues having specific activity approaching that of the free valine, such as would be possessed by an intermediate of protein synthesis present in such small quantity. To test this would require more elaborate fractionation. It would also be desirable to try to make a more detailed study of the kinetics of labelling of the valine residues in such a fraction



(*a*) (*b*)

Fig. 2. Radioautographs of extracted residues (fraction 1) of Italian ryegrass after administration of L-[^{14}C]valine (see text): (*a*) preflowering stage; (*b*) flowering stage. (×0·41.)

under conditions of rapid increase and decrease of the specific activity of the free valine.

The action of ether–water at room temperature resulted in a doubling or trebling of the amphoteric forms of bound amino acids and bound valine in fractions 6 and 8 when compared with the action of boiling water (expts. III and IV). Since there was no parallel increase in the percentage of radioactivity entering the valine of fraction 8 it is reasonable to conclude that autolysis of pre-existing protein during the ether–water treatment caused the increase in this peptide-like

Table 1. *Distribution of dry matter and nitrogen in fractions from grass*

Expt.	Dry matter (% of total dry matter of grass)				Nitrogen (% of dry matter of fraction)				Nitrogen (% of total N of grass)			
Fraction	C	II	III	IV	C	II	III	IV	C	II	III	IV
Original grass	100	100	100	100	5·0	2·8	3·2	2·9	100	100	100	100
Residues (1)	62·6	58·4	68·3	63·1	5·8	4·1	4·0	3·8	71·4	85·9	85·4	82·4
Acetic acid washings (2)	3·1	5·2	4·5	17·9	1·9	0·8	2·2	1·5	1·2	1·5	3·1	9·0
Diffusate (3)	32·2	31·6	24·1	17·3	3·8	1·0	1·4	1·3	24·0	11·4	10·5	7·8
Phenol extract (4)	8·0	3·3	3·1	1·6	3·1	1·6	1·6	3·6	5·0	1·9	1·6	2·0
Middle compartment (5)	2·7	1·2	1·4	0·5	1·7	2·8	0·7	1·3	0·89	0·92	0·33	0·23

Table 2. *Distribution of carboxyl nitrogen and valine nitrogen in fractions from grass*

Expt.	Nitrogen (% of total N of grass)				
	C	I	II	III	IV
Valine N of hydrolysed residues (1)	2·8	3·5	4·9	4·8	4·2
Carboxyl N after hydrolysis of fraction 5	0·15	—	0·083	0·095	0·060
Valine N after hydrolysis of fraction 5	0·00096	0·0103	0·0036	0·0017	0·0012
Carboxyl N before hydrolysis of fraction 6	1·36	—	0·35	0·21	0·22
Carboxyl N after hydrolysis of fraction 6	1·75	—	0·40	0·35	0·50
Carboxyl N of bound amino acids of fraction 6 (by diff.)	0·39	—	0·05	0·14	0·28
Free valine N of fraction 7*	0·130†	0·130*	0·105*	0·044*	0·038*
Bound valine N after hydrolysis of fraction 8	0·0043	0·0154	0·0054	0·0074	0·0270

* Corrected values (see text). † By isotope dilution (see text).

Table 3. *Recovery of radioactivity in valine residues of fractions from grass as a percentage of that initially administered as* [^{14}C]*valine*

Expt.	Recovery (%)			
	I	II	III	IV
Free valine (fraction 7)	38·7	53·5	25·9	15·4
Protein (fraction 1)	13·0	21·0	54·1	46·8
Bound valine (fraction 5) (acidic and neutral)	0·29	0·018	0·013	0·010
Bound valine (fraction 8) (amphoteric)	0·77	0·088	0·32	0·37
Total recovery	52·8	74·6	80·3	62·6

Table 4. *Specific radioactivity of valine residues of fractions from grass as a percentage of the specific radioactivity of the free valine of the grass at the end of the experiment*

Expt.	Specific radioactivity (%)			
	I	II	III	IV
Protein (fraction 1)	1·2	0·8	1·9	2·7
Acidic and neutral bound amino acids (fraction 5)	9·5	1·0	1·3	2·0
Amphoteric bound amino acids (fraction 8)	16·8	3·2	7·3	3·4

material. The neutral and acidic bound amino acids in fraction 5 did not increase. The implications of these observations for the studies of Carnegie (1961*b*) are discussed by him in that paper.

The concentration of free valine found in the grass samples agrees fairly well with that found by Kemble & Macpherson (1954*b*). Neither the free valine nor the total free amino acids of fraction 6 seemed to increase as a result of the ether–water treatment. The valine content of the grass protein was also close to the values found by Kemble & Macpherson (1954*a*).

Miettinen (1959) has studied the metabolism by pea plants of DL-[*carboxy*-^{14}C]alanine. He usually administered this through the roots, and found some incorporation of [^{14}C]alanine into fractions probably similar to our fractions 5 and 8. Incorporation of ^{14}C into the neutral and anionic

fraction was particularly extensive; however, residues other than alanine, such as simple sugars, accounted for some of this. Nevertheless, much of the activity (in roots only) was present as alanine residues in compound 'A'. A somewhat similar compound was formed in roots from DL-[^{14}C]valine. Dr J. K. Miettinen (personal communication) informs us that substance 'A' results from administration of DL-[^{14}C]alanine but not of L-[^{14}C]-alanine.

Linko (1958) found rapid labelling of a number of compounds, some of which were of low molecular weight and contained bound amino acids, on infiltrating leaves of *Phlox decussata* and *Asplenium septentrionale* with [^{14}C]pyruvate and [^{14}C]glutamate.

The present experiments demonstrate that L-[^{14}C]valine of high specific activity introduced in the transpiration stream can be fairly rapidly and extensively incorporated into protein distributed right through the severed portion of the plant, perhaps without much disturbance of the normal metabolic processes. We hope that these observations will prove useful for planning future studies of the biosynthesis of proteins in plants.

SUMMARY

1. Uniformly labelled L-[^{14}C]valine of high specific activity was administered to severed shoots of Italian ryegrass in the transpiration stream and extensive incorporation of labelled valine residues into protein resulted. The distribution of labelled protein in the plant was observed by radioautography.

2. The specific activities acquired under these conditions by valine residues in bound non-protein form suggest that most of such compounds are not directly concerned in the biosynthesis or the catabolism of protein.

3. Extraction with ether–water resulted in some catabolism of protein to peptides not present when the tissue was extracted by boiling water.

4. A non-volatile radioactive impurity, presumably a product of radiochemical decomposition, was present in the [^{14}C]valine as purchased.

5. Evidence was obtained for the occurrence of a metastable tautomeric form of *N*-2:4-dinitrophenyl-L-valine exhibiting mutarotation in acetic acid.

We have had valued advice and help with various aspects of this work from Mr D. J. Benzie, Mr P. R. Carnegie, Dr J. R. Catch, Dr H. G. Jones, Dr E. I. McDougall, Professor H. K. Porter and Dr T. S. Work. We are also grateful to Miss C. J. Blair for technical assistance.

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Fractionation of the β-Glucosidases from *Aspergillus niger*

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(*Received* 9 *May* 1960)

In common with many saprophytic moulds *Aspergillus niger* produces in its culture medium a complex mixture of polysaccharide- and protein-depolymerizing enzymes. The commercial preparation (E19) of the extracellular enzymes of *A. niger* has been shown to hydrolyse cellulosic substrates, yeast glucan, laminarin, xylan, starch, polygalacturonic acid and gelatin (Walseth, 1952*a*, *b*; Holden & Tracey, 1950; Whistler & Smart, 1953; Stone, 1957*a*; Wolf, Jurkovich & MacMasters, 1959). The β-glucosidases of *A. niger* active on β-linked glucose disaccharides were investigated by Crook & Stone (1957). It was shown that during the hydrolysis of cellobiose, a possible intermediate in the enzymic hydrolysis of cellulose, the transient formation of oligosaccharides by transglucosylation occurred. Before attempting any study of the action patterns of the β-glucosidases from *A. niger* that hydrolyse cellulose it was therefore desirable to distinguish and if possible to separate them from the β-glucosidases studied earlier. Preliminary experiments, which have been briefly reported (Stone, 1957*a*), showed that calcium phosphate could reversibly adsorb the *A. niger* enzymes, and, subsequently, synthetic ion-exchange resins were also found satisfactory as adsorbents for elution chromatography. Details of the fractionation experiments are now reported together with some data on the specificity of the fractionated components.

MATERIALS AND METHODS

Substrates. Salicin and cellobiose were from L. Light and Co. Ltd. Cellopentaose was prepared and donated by Dr D. R. Whitaker, National Research Laboratories, Ottawa, Canada. Cellodextrin was prepared by ethanol fractionation of a cellulose hydrolysate. The average degree of polymerization (av. D.P.) as determined by iodometric titration (Martin, Smith, Whistler & Harris, 1941) was 80. Sodium carboxymethylcellulose, Edifas 'B' (controlled metals content, normal viscosity, 0·45–0·55 degree of substitution) was a product of Imperial Chemical Industries Ltd., Steventon, Ayrshire, Scotland. Phosphoric acid-swollen cellulose was prepared according to Walseth (1952*a*) from cotton lint (American card sliver) obtained from the Shirley Institute, Didsbury, Manchester; the D.P. determined by the British Rayon Research Association from the intrinsic viscosity of the nitrate according to Immergut, Rånby & Mark (1953) was 2059. Soluble laminarin from *Laminaria digitata* was purchased from the Institute of Seaweed Research, Inveresk, Scotland. Lichenin was extracted from the dried fronds of *Cetraria icelandica* (Iceland moss) purchased from Brome and Schimmer (7, Leather Market, London, S.E. 1). The method used was that of Hess & Lauridsen (1940) described by Meyer & Gürtler (1947); the final stages were as described by Drake (1942). The light-brown product, dried at 60° *in vacuo*, contained 96·2% of polyglucose.

Enzyme preparation. The commercial preparation E19 (Rohm and Haas Co., Philadelphia 5, Penn., U.S.A.), an ethanol-precipitated culture filtrate of *Aspergillus niger*, was supplied as a stable powder. Stock batches of the enzyme were prepared by allowing a 10% (w/v) suspension of the powder in water to stand for several hours in a refrigerator with occasional stirring. The insoluble material was spun off and the dark-brown supernatant dialysed against distilled water in cellulose nitrate sacs (Stone, 1957*b*) until free of reducing sugars and inorganic salts. The solutions were then freeze-dried and the powders stored in a desiccator under vacuum. These powders were readily soluble in water and will be referred to as the crude enzyme. The carbohydrate content determined as glucose by the orcinol method (Pirie, 1936) was 45·5% and by the anthrone method (Trevelyan & Harrison, 1952) was 19·5%. The protein content, determined by the Folin biuret method with bovine serum albumin as standard (Lowry, Rosebrough, Farr & Randall, 1951), was 63·5% and based on Kjeldahl-nitrogen determination was 47·3%.

Enzyme assays. Enzyme activity on all substrates was measured by determining reducing sugar liberated under standard conditions. In all cases the pH of the reaction mixture was 5·0 and the incubation temperature 40°. Reactions were stopped by mixing 1 ml. of the reaction mixture with 1 ml. of $ZnSO_4$ soln. in a 15 ml. centrifuge tube and rapidly adding 1 ml. of $Ba(OH)_2$ (Somogyi, 1945). After centrifuging, samples (1 ml.) of the supernatant were removed to 25 ml. Folin–Wu tubes and the reducing sugar was determined as glucose by the Nelson (1944) colorimetric method with the Somogyi (1952) alkaline copper reagent. Light-absorption was measured in a colorimeter (A. Gallenkamp and Co. Ltd., London) with an Ilford 204 filter.

The reaction mixtures incorporating the individual substrates had the following compositions. (*a*) Salicin: 0·5 ml. of 0·5% salicin, 0·4 ml. of 0·1 M-acetate buffer, 0·2 ml. of enzyme solution; incubation time 1 hr. (*b*) Cellopentaose: 0·8 ml. of 0·06% cellopentaose in 0·1 M-acetate buffer, 0·2 ml. of enzyme solution; incubation time 1 hr. (*c*) Cello-

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dextrin: 0·8 ml. of 0·25% cellodextrin in 0·1 M-acetate buffer, 0·2 ml. of enzyme solution; incubation time 2 hr. (*d*) Sodium carboxymethylcellulose (SCMC): 0·7 ml. of 0·75% SCMC, 0·2 ml. of 0·2 M-acetate buffer, 0·1 ml. of enzyme solution; incubation time 2 hr. Since partially swollen fibres could be seen with the microscope in simple aqueous solutions of the SCMC used, complete dispersion of the SCMC for use in enzyme assays was obtained by the following procedure; 500 ml. of an approx. 5% (w/v) solution was prepared by swelling SCMC in 200 ml. of water overnight and stirring in the remaining 300 ml. of water with a high-speed stirrer. Enough 40% NaOH was added to bring the NaOH concentration to 5%; the mobile solution was neutralized with 0·5 N-HCl and the NaCl removed by dialysis against distilled water. The final SCMC concentration was determined by dry weight and the SCMC substrate solution prepared by dilution. (*e*) Phosphoric acid-swollen cellulose: 12·5 mg. of swollen cellulose, 2 ml. of 0·2 M-acetate buffer, 2 ml. of enzyme solution; incubation in 25 ml. conical flasks for 5 hr. with shaking at 60 strokes/min. (*f*) Laminarin: 0·6 ml. of 0·25% laminarin in 0·1 M-acetate buffer, 0·4 ml. of enzyme solution; incubation time 2 hr. (*g*) Lichenin: 0·5 ml. of 2% (w/v) lichenin dispersion in 0·2 M-acetate buffer made by heating to 60°, 2 ml. of enzyme solution; incubation time 5 hr.

Calcium phosphate columns. Calcium phosphate (Brushite modification) was prepared according to Tiselius (1954). A total precipitation time of 1·5 hr. gave a product with suitable packing properties. After washing by decantation six times with 5 mM-Na_2HPO_4 the precipitate was stored under the same solution.

Columns were packed by pouring a suspension of calcium phosphate on to a glass-wool pad and allowing the calcium phosphate to settle under gravity, excess of suspending solution then being run off. When sufficient calcium phosphate had been added the bed was packed down under slight positive pressure of air (about 3 lb./in.²). The column was then washed until equilibrated with phosphate buffer of desired pH.

Dowex-1 columns. Dowex-1 X 4, 200–400 mesh, a quaternary-ammonium anion-exchange resin, was used in all experiments. Before packing a column the resin was converted into the hydroxyl form with 2 N-NaOH and then into the chloride form with N-HCl and was washed on a Büchner funnel to remove excess of chloride. The suspended resin was deaerated (cf. Boman & Westland, 1956) in a Büchner flask and the column packed by pouring the suspension on to a glass-wool pad. After washing with water until the chloride test was negative the column was equilibrated with the desired buffer.

Elution procedure. All chromatographic experiments reported were conducted at room temperature. The enzyme was applied to the column as a solution in a small volume of the same buffer as was used to equilibrate the column. The enzyme was eluted from the calcium phosphate columns with a linearly increasing concentration of phosphate at a constant pH and from Dowex-1 columns by a linearly increasing concentration of chloride in 2-amino-2-hydroxymethylpropane-1:3-diol–HCl (tris–HCl) buffer at constant pH. The linear gradients were obtained with the mixing device described by Parr (1954). The flow rate from the columns was controlled by a tungsten-needle valve. Fractions were collected automatically in equal volumes (Crook & Datta, 1951). The linearity of the gradients was checked by phosphate (Allen, 1940) or chloride (Kolthoff & Sandell, 1952) analyses of the fractions. The protein content of the fractions was estimated by measuring E at 280 mμ in a Uvispek spectrophotometer. In some experiments carbohydrate concentrations of the fractions were measured by the orcinol method.

RESULTS

Elution analysis of Aspergillus niger *preparation on Dowex-1 columns*

A sample (15 mg.) of the crude enzyme dissolved in 5 mM-tris–HCl, pH 7·0, was applied to a column (67 cm. × 0·7 cm.) of Dowex-1 equilibrated with 5 mM-tris–HCl, pH 7·0. Elution was effected with 90 ml. of 5 mM-tris–HCl, pH 7·0, followed by a concentration gradient of chloride (mixing vessel: 250 ml. of 5 mM-tris–HCl, pH 7·0; reservoir: 250 ml. of 0·75 M-NaCl in 5 mM-tris–HCl, pH 7·0). Fractions (7·5 ml.) were collected at a flow rate of 0·4 ml./min. and stored at 5°.

Fig. 1 (*a–c*) shows the elution pattern of hydrolytic activity towards cellobiose, cellopentaose,

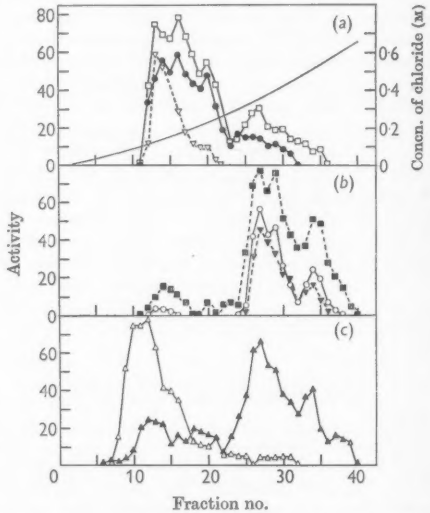

Fig. 1. Chromatography of 15 mg. of crude enzyme (7 mg. of protein) on a Dowex-1 column (67 cm. × 0·7 cm.) by gradient elution with sodium chloride, 0·005–0·75 M, in 5 mM-tris–HCl buffer, pH 7·0. Activity towards cellobiose (●), cellopentaose (□), salicin (▽), cellodextrin (■), SCMC (○), swollen cellulose (▼), lichenin (▲) and laminarin (△), protein concentration (not shown) and chloride concentration (continuous line) were determined on each fraction as described in the text. The enzyme activities are expressed as μg. of reducing sugar (as glucose) liberated under standard conditions/ml. of deproteinized assay solution.

cellodextrin, SCMC, alkali-swollen cellulose, laminarin, lichenin and salicin in the same experiment. Protein was eluted in the initial 5 mM-tris–HCl fractions and was accompanied by a very low activity towards laminarin but no activity towards the other substrates. Protein was eluted throughout the chloride gradient but little correlation between protein content and the enzymic activities of the fractions was apparent. Approximately 50 % of the orcinol-reacting carbohydrate was eluted by the 5 mM-tris–HCl fractions; the remainder was eluted in the chloride gradient. There was no correlation between carbohydrate content of the fractions and their enzymic activities.

Inspection of the elution patterns of hydrolytic activity towards the polymeric series of β-1:4-glucosidic substrates suggests the presence of two different groups of components, a group eluted by chloride concentrations in the range 0·1–0·3 M and a group eluted in the range 0·3–0·6 M. The hydrolysis of cellobiose and cellopentaose is high in the first group and low in the second group, whereas the reverse is true of the hydrolysis of cellodextrin, SCMC and swollen cellulose; in fact there was no detectable hydrolysis of swollen cellulose by the 0·1–0·3 M-chloride components and the activity towards SCMC was very weak.

Fig. 1 (*a*) shows that salicin hydrolysis was confined to those fractions which hydrolyse cellobiose and further that not all the fractions which hydrolyse cellobiose were equally active in hydrolysing salicin. Only very weak hydrolysis of laminarin was shown by the fractions eluted in the 0·3–0·6 M-chloride region. Almost all the activity towards soluble laminarin was eluted by chloride below 0·22 M (Fig. 1*c*) and thus at least part of this activity was due to components different from those which hydrolyse cellobiose and cellopentaose. Fractions which hydrolyse lichenin appeared in both the 0·1–0·3 M- and the 0·3–0·6 M-chloride region although the activity was higher in the latter.

Elution analysis of Aspergillus niger *enzymes on calcium phosphate columns*

Preliminary experiments under the conditions reported earlier (Stone, 1957*a*) showed that as the pH was lowered the molarity of phosphate required to elute completely all the activity towards SCMC was raised. Thus 0·045 M-phosphate was required at pH 7·4, 0·055 M at 6·8, and 0·09 M at 5·8. There was a corresponding improvement in the resolution of the components as the pH was lowered. Resolution was further improved by using columns at least three times as long as those used in earlier experiments (Stone, 1957*a*).

A sample (15 mg.) of the crude enzyme dissolved in 5 mM-phosphate buffer, pH 5·8, was applied to a column 65 cm. × 0·7 cm. equilibrated with 5 mM-phosphate buffer, pH 5·8. Elution was effected under slight positive pressure with 120 ml. of 5 mM-phosphate buffer, pH 5·8, followed by a phosphate-concentration gradient (mixing vessel: 450 ml. of 5 mM-phosphate, pH 5·8; reservoir: 450 ml. of 0·15 M-phosphate, pH 5·8). Fractions (7·5 ml.) were collected at a flow rate of 0·4 ml./min. and stored at 5°.

Figs. 2 (*a*, *b*) and 4*a* show the distribution among the fractions of activity toward cellobiose, cellopentaose, cellodextrin, SCMC, swollen cellulose and laminarin. Solutions of the crude enzyme preparation are dark brown. The pigment responsible was adsorbed at the top of the column and was not eluted by 0·15 M-phosphate buffer, so that with the exception of some of the first 5 mM-phosphate fractions the eluate was water-clear. In preliminary experiments carbohydrate (orcinol) determination showed that 60 % of the total carbohydrate was eluted in the 5 mM-phosphate fractions. The level of carbohydrate in the fractions eluted by the phosphate gradient was on the limits of sensitivity of the method. Protein (17 % of the total recovery) was eluted in the 5 mM-phosphate fraction and these fractions showed a low activity towards all

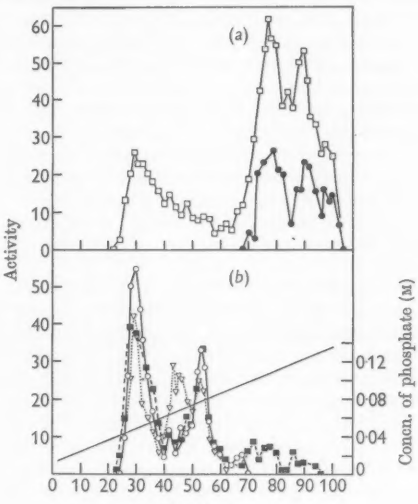

Fig. 2. Chromatography of 15 mg. of crude enzyme (7 mg. of protein) on a 67 cm. × 0·7 cm. calcium phosphate column by gradient elution with phosphate buffer, 5–150 mM, pH 5·8. Activity towards cellobiose (●), cellopentaose (□), cellodextrin (■), SCMC (○), swollen cellulose (▽), protein concentration (not shown) and phosphate concentration (continuous line) were determined on each fraction as described in the text.

substrates except cellobiose and cellodextrin. Protein was eluted throughout the phosphate gradient but little correlation between the protein content of the fractions and their enzymic activity was apparent. As in the chromatography on Dowex-1, the elution pattern of activity towards the polymeric series of β-1:4-glucosidic substrates suggests the presence of two different groups of components, a group eluted by phosphate concentrations in the range 0·04–0·09 M and a second in the range 0·09–0·14 M. The first group shows high activity towards the cellulosic substrates and some activity towards cellopentaose but does not hydrolyse cellobiose. The second group shows high activity towards cellobiose and cellopentaose, a low activity towards cellodextrin but no activity towards SCMC or swollen cellulose. Soluble laminarin (Fig. 4*a*) was hydrolysed by fractions eluted by 0·02–0·06 M-phosphate and also by fractions eluted by 0·10–0·12 M-phosphate.

It is significant that the two groups of components hydrolysing the β-1:4-glucosidic link appear to have retained their identities, as judged by their relative activities on the cellosaccharides and cellulosic substrates, under the different conditions prevailing on the two adsorbents. These differences in conditions are reflected in the reversal of the elution order of the two groups in going from one adsorbent to the other. The components hydrolysing laminarin are eluted in the low-molarity chloride fractions on Dowex-1 and partially in the low and partially in the high-molarity phosphate fractions from calcium phosphate. These differences in elution order on the two adsorbents were exploited to obtain a separation of the two groups of components hydrolysing β-1:4-glucosidic linkages from one another and partially from components hydrolysing β-1:3-glucosidic linkages.

Preparative chromatography on Dowex-1

In order to accumulate sufficient material for rechromatography several preparative-scale experiments were run with Dowex-1. In these experiments 1 g. of crude enzyme, dissolved in 5 mM-tris–HCl, pH 7·0, was applied to a column (73 cm. × 3·8 cm.) of Dowex-1 equilibrated with the same buffer. Elution was effected with 750 ml. of 5 mM-tris–HCl followed by a concentration gradient of chloride (mixing vessel: 1500 ml. of 5 mM-tris–HCl, pH 7·0; reservoir: 1500 ml. of 0·75-M-NaCl in tris–HCl, pH 7·0). Fractions (20 ml.) were collected and assayed for activity towards cellobiose, SCMC and laminarin. The elution patterns closely resembled those found for the analytical columns (Fig. 3*a*). The fractions were pooled to give two solutions, one containing the components eluted by 0·1–0·3 M-chloride and the other the components eluted by 0·3–0·6 M-chloride. These two solutions were freeze-dried and reconstituted in a small volume of water and dialysed in cellulose nitrate sacs for 48 hr. against frequently changed distilled water. The small amount of material precipitated during dialysis was centrifuged off and the clear solutions were stored in the frozen condition.

Rechromatography of fractions separated on Dowex-1

With Dowex-1. A portion of the solution containing the 0·1–0·3 M-chloride components was dialysed overnight against 5 mM-tris–HCl. A sample (0·3 ml.; 10·7 mg. of protein) of the equilibrated solution was chromatographed under the same conditions as previously described for the crude enzyme. The elution pattern of the activity against cellobiose, SCMC and soluble laminarin is shown in Fig. 3 (*b*). The result of a similar experiment with 0·6 ml. (7·6 mg. of protein) of the 0·3–0·6 M-chloride fraction is shown in Fig. 3 (*c*).

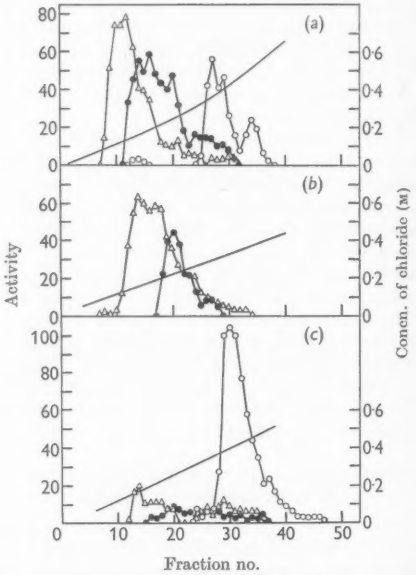

Fig. 3. Fractions from preparative chromatography on Dowex-1 rechromatographed on Dowex-1 columns (67 cm. × 0·7 cm) by gradient elution with tris–HCl buffer, 0·005–0·75 M, pH 7·0. Activity towards cellobiose (●), laminarin (△), protein concentration (not shown) and SCMC (○), chloride concentration (continuous lines) were determined on each fraction. (*a*) Elution diagram of 15 mg. of crude enzyme (7 mg. of protein) included for comparison. (*b*) Elution diagram of 0·3 ml. of concentrated 0·1–0·3 M-chloride fractions (10·7 mg. of protein). (*c*) Elution diagram of 0·6 ml. of concentrated 0·3–0·6 M-chloride fractions (7·6 mg. of protein).

It is apparent that the fractionated components are eluted at approximately the same point in the chloride gradient as in the chromatography of the unfractionated enzyme. The 0·1–0·3 M-chloride fraction is free from all activity towards SCMC but by comparison with the elution pattern of the whole enzyme it appears to lack some of the components which hydrolyse cellobiose. The 0·3–0·6 M-chloride fraction contains the SCMC-hydrolysing activity together with a small amount of activity towards cellobiose and laminarin, some of which appears in the low chloride concentration region. This contamination is most probably due to 'tailing' of the cellobiose- and laminarin-hydrolysing components normally eluted in the 0·1–0·3 M-chloride fractions into the later fractions on the preparative columns.

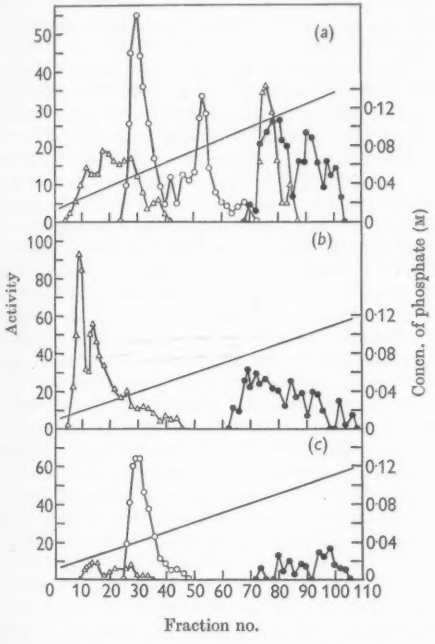

Fig. 4. Fractions from preparative chromatography on Dowex-1 rechromatographed on calcium phosphate columns by gradient elution with phosphate buffer, 0·005–0·15 M, pH 5·8. Activity towards cellobiose (●), SCMC (○), laminarin (△), protein concentration (not shown) and phosphate concentration (continuous lines) were determined on each fraction. (*a*) Elution diagram of 15 mg. of crude enzyme (7 mg. of protein). (*b*) Elution diagram of 3·5 ml. of concentrated 0·1–0·3 M-chloride fractions (12·5 mg. of protein). (*c*) Elution diagram of 0·6 ml. of concentrated 0·3–0·6 M-chloride fractions (7·6 mg. of protein).

With calcium phosphate. A portion of the solution containing the 0·1–0·3 M-chloride components was dialysed overnight against 5 mM-phosphate buffer, pH 5·8, and 0·35 ml. (12·5 mg. of protein) of the equilibrated solution was applied to a column 65 cm. × 0·7 cm. and chromatographed under the same conditions as previously described for the crude enzyme. The results of this experiment and a similar one with 0·6 ml. (7·6 mg. of protein) of 0·3–0·6 M-chloride fraction is shown in Fig. 4 (*b*, *c*). On rechromatography on calcium phosphate the 0·1–0·3 M-chloride fraction showed only components hydrolysing laminarin and cellobiose, in agreement with the results obtained on Dowex-1 rechromatography. However, in this case there has been a complete resolution of the laminarin- and cellobiose-hydrolysing components. The rechromatographed 0·1–0·3 M-chloride fraction lacks the activity towards laminarin that was eluted in the 0·09–0·12 M-region in the chromatography of the crude enzyme. The 0·3–0·6 M-chloride fraction showed only SCMC-hydrolysing activity together with a small amount of activity towards laminarin and cellobiose. The second major SCMC-hydrolysing component present in the 0·07–0·09 M-phosphate fractions of the crude enzyme was absent in the rechromatographed 0·3–0·6 M-chloride fraction.

From these experiments it is clear that the appearance of activity towards cellobiose, laminarin and SCMC in different fractions on Dowex-1 chromatography is not caused by the chromatographic procedure itself, and further that the active fractions obtained by calcium phosphate chromatography can be identified with those obtained by chromatography on Dowex-1.

Hydrolysis of lichenin

Chromatography on Dowex-1 (Fig. 1*c*) shows clearly that those fractions eluted by 0·3–0·6 M-chloride with activity towards the cellulosic substrates also hydrolysed lichenin. However, it is not possible to associate the lichenin-hydrolysing activity in the 0·1–0·3 M-chloride fractions exclusively with either the laminarin- or cellodextrin-hydrolysing components since the activity towards lichenin extends to fractions active towards both of these substrates.

DISCUSSION

Comparison of components hydrolysing β-1:4-glucosidic linkages

The results of the elution analyses of the *Aspergillus niger* preparation indicate the presence of two different groups of components hydrolysing β-1:4-glucosidic linkages. The difference in specificity between these two groups at first sight would appear to depend on whether the β-1:4-glucosidic

linkage was available in a substrate of a low or a high degree of polymerization (D.P.). Two fractions with similar apparent specificities have been obtained by Grassman, Zechmeister, Tóth & Stadler (1933) by treating an *Aspergillus oryzae* (Luitzym) preparation with aluminium metahydroxide at pH 3·5. Two successive treatments gave a preparation, which was free of activity towards cellobiose, that slightly hydrolysed cellotetraose, but which actively hydrolysed cellohexaose, cellodextrin (av. D.P. 9) and sulphuric acid-treated cellulose ('hydratcellulose'). Treatment of the aluminium metahydroxide with 0·04M-sodium bicarbonate eluted material which actively hydrolysed cello-biose, -triose, -tetraose and -hexaose, whereas the cellodextrin and cellulose were only weakly attacked. Grassman *et al.* (1933) called the unadsorbed fraction a 'polysaccharase' and the eluted fraction an 'oligosaccharase'. The two groups of components now identified in the *A. niger* preparation would fit this classification, although the overlap in activity in the cellosaccharide range is different.

A nomenclature for these enzymes based on their action patterns (cf. Jermyn, 1957) would be more significant but the fractionation experiments themselves do not allow any direct conclusions in this respect. It is very likely, however, that the polysaccharase components of both *A. oryzae* and *A. niger* resemble the cellulose-hydrolysing enzymes of *Myrothecium verrucaria* (Whitaker, 1957) and of *Stachybotrys atra* (Thomas, 1956) in having an endoglucosidase action pattern. This is supported by the evidence of Husemann & Lotterle (1950), who have shown by physical methods that the *A. oryzae* (Luitzym) preparation attacks cellulose in a random fashion characteristic of an endoglucosidase, and the appearance of a series of oligosaccharide intermediates during the hydrolysis of cellodextrins by the *A. niger* preparation suggests that it also possesses an endo-β-1:4-glucosidase (Stone, 1954). The striking correlation in the present study between the elution positions of the components hydrolysing lichenin and those active against cellulosic substrates suggests that the same enzymes are responsible. Moreover, the ability of these β-1:4-glucosidases to hydrolyse lichenin also suggests their endoglucosidase nature. The most recent work on the structure of lichenin shows that it is a β-glucan with about 70% 1:4-linkages and 30% 1:3-linkages (Chanda, Hirst & Manners, 1957; Peat, Whelan & Roberts, 1957), and that these are arranged in the repeating sequence –Gβ–4Gβ–4Gβ–3– (Peat *et al.* 1957). The molecule is believed to be entirely linear and to have an av. D.P. of 63 (Manners, 1959). If this lichenin structure is valid then an exo-β-glucosidase hydrolysing only 1:4-glucosidic linkages and detaching glucose units singly or in pairs from the chains might be blocked by the 1:3-links. An endo-β-1:4-glucosidase, on the other hand, would be able to hydrolyse at least one of the two β-1:4-glucosidic linkages in the cellotriosyl residues of the chain.

The identity of this fraction of the 'lichenase' activity with the 'cellulase' activity of *A. niger* is in agreement with the findings of Otto (1929), who was unable to separate the two activities from barley malt with a series of adsorbents. However, other workers have concluded that 'lichenase' and 'cellulase' are different enzymes (Ullman, 1932; Freudenberg & Ploetz, 1939; Ploetz, 1939).

The substrate range of the cellosaccharide-hydrolysing components is comparable with that of the intracellular exo-β-glucosidase of *S. atra* described by Youatt (1958), which removed single glucose residues from cellosaccharides with D.P. values at least up to 11, and to the exo-β-glucosidase of almond emulsin (Peat, Thomas & Whelan, 1952) reported by Grassman *et al.* (1933) to have the same substrate range as the *A. oryzae* (Luitzym) 'oligosaccharase'.

The apparent chain-length specificities of exo- and endo-glucosidases can be regarded as a consequence of their action patterns. Exoglucosidase action, other things being equal, will depend on the concentration of terminal glucosidic linkages; thus for equal concentrations of substrate the low D.P. cellosaccharides would provide the highest concentration of hydrolysable β-glucosidic linkages.

It is not known if there is an upper D.P. limit for the exoglucosidases hydrolysing cellosaccharides, i.e. if their action is independent of chain length as with β-amylase (Manners, 1954) and gluca-amylase (Phillips & Caldwell, 1951), or if as the substrate D.P. increases the affinity for the enzyme decreases and becomes effectively zero at a certain D.P. [cf. *Saccharomyces cereviseae* maltase (Whelan, 1953; Phillips, 1959)]. The action of endoglucosidases is generally independent of substrate length above a certain D.P., the value of which varies with the type and source of the enzyme, e.g. above 4 for the cellulose-hydrolysing enzymes of *Myrothecium verrucaria* (Whitaker, 1954), above 3 for salivary α-amylase (Manners, 1954), above 7 for *Lactobacillus bifidus* dextranase (Bailey & Clarke, 1959). The relatively low activity of the *A. niger* 'polysaccharase' components on the lower D.P. cellosaccharides is thus consistent with an endoglucosidase action pattern.

Linkage specificity of the β-glucosidase components

Ploetz & Pogacar (1949), using a crude and a fractionated *A. oryzae* (Luitzym) preparation, studied the kinetics of the hydrolysis of three β-glucans, cellulose, yeast glucan and luteose, in which the main inter-glucose linkages are of the β-1:4-, β-1:3- and β-1:6-glucosidic type respectively.

They chose to interpret their results in terms of the presence of a non-specific oligosaccharase and polysaccharase rather than in terms of specific β-1:4-, β-1:3- and β-1:6-glucosidases. More recent experiments have suggested that specific β-1:3- and β-1:4-glucosidases do exist. Duncan, Manners & Ross (1956) showed that the ability of the extract of the alga *Cladophora rupestris* to hydrolyse laminarin, a predominantly β-1:3-linked glucan, was lost on heating it at 60° for 15 min. whereas the cellodextrin-hydrolysing activity was retained. Aitken, Eddy, Ingram & Weurman (1956) made similar observations on the hydrolysis of laminarin and cellulosic substrates by *M. verrucaria* filtrates before and after heating at 60° for 10 min. Their observations are, however, at variance with those of Kooiman (1957), who observed considerable laminarin hydrolysis by *M. verrucaria* filtrates after heating for 3 min. at 100°. Finally, Reese & Mandels (1959) have observed laminarin hydrolysis by culture filtrates of a number of fungi in the absence of detectable hydrolysis of cellulosic substrates. Furthermore there is evidence based on the paper-chromatographic identification of the end products of prolonged enzymic action that hydrolytic activity towards laminarin may be either the result of endo-β-1:3-glucosidase activity (algal enzymes: Manners, 1955; fungal enzymes: Reese & Mandels, 1959) or by exo-β-glucosidase action (almond emulsin: Peat *et al.* 1952; culture filtrates of basidiomycete QM 806 and *Sporotrichum pruinosum*: Reese & Mandels, 1959). MacWilliam & Harris (1957) fractionated barley-malt enzymes on alumina and concluded from the identity of the products of hydrolysis of laminarin by the fractions that both endo- and exo-glucosidases hydrolysing β-1:3-glucosidic links were present. The exo-glucosidase activity was eluted in the same fractions as some of the cellobiose-hydrolysing activity but none of these fractions hydrolysed salicin.

The elution patterns of the *A. niger* preparation chromatographed on Dowex-1 and calcium phosphate show directly that the fractions with activity towards the β-1:3-glucosidic linkages in laminarin are distinct from those with activity towards cellulosic substrates. As reported earlier (Stone, 1957*a*), the activity towards β-1:3-glucosidic linkages can be resolved into two distinct groups of components by chromatography on calcium phosphate. At present no statement can be made about the action patterns of these two groups of components, but it is relevant that the more strongly adsorbed components appear in fractions which also hydrolyse cellobiose. Coincidence of laminarin- and cellobiose-hydrolysing activity is also apparent in the Dowex-1 elution experiments. Whether this coincidence in elution position is due to the presence in these fractions of linkage-specific β-glucosidases with similar physical properties or to the presence of exo-β-glucosidases whose specificities are not directed towards the position of the glucosidic linkage in the substrate remains to be determined. It will be noted, however, that all the cellobiose-hydrolysing activity is not associated with activity towards laminarin.

Salicin- and cellobiose-hydrolysing activity appear in the same fractions in Dowex-1 experiments but the relative activities of the fractions on these two substrates is variable. Again this may reflect the presence of several β-glucosidases with different affinities for salicin and cellobiose or separate specific enzymes with similar elution characteristics. Zechmeister, Tóth & Fürth (1940) have separated the cellobiose- and salicin-hydrolysing components from almond emulsin. A distinction between aryl β-glucoside- and cellobiose-hydrolysing enzymes has been made by other workers (Jermyn, 1957; Hash & King, 1958).

Nature of the components within each group

The elution patterns for both Dowex-1 and calcium phosphate show that for each of the β-glucosidases the activity is distributed among a number of components which may or may not be completely resolved under the experimental conditions. Among the components apparent in the two groups of fractions hydrolysing β-1:4-glucosidic linkages there generally appears to be a consistent ratio of activities towards the polymer homologous series of substrates. This is not, however, true of the component eluted at 0·06–0·07 M-phosphate (Fig. 2*b*), which has apparently a much higher activity towards swollen cellulose than towards the lower D.P. cellulosic substrates (Halliwell, 1957).

Different forms of proteins with similar biological activity have been encountered by other workers during chromatography, e.g.: insulin (Dickinson, 1956); ribonuclease (Tanford & Hauenstein, 1956); phosphodiesterase (Boman, 1955); lysozyme (Tallan & Stein, 1953); enolase (Malmström, 1957); horse-radish acid phosphatase (Boman & Westland, 1956); pea phosphatase and metaphosphatase (Pierpoint, 1957). Several explanations for these components are possible. They may represent polymeric forms of a single protein. Tiselius, Hjertén & Levin (1956) have shown that R- and C-phycocyanin yield two components in chromatography on calcium phosphate at pH 6·3. These phycocyanins are known to dissociate into half molecules in the range pH 6·2–6·8. Changes in the protein molecules due to hydrolysis of peptide or amide bonds have been shown to occur without loss of biological activity [e.g. insulin (Harfenist, 1953); ribonuclease (Tanford & Hauenstein, 1956); lysozyme (Tallan & Stein, 1953), papain (Finkle & Smith, 1958); phosphodiesterase (Björk & Boman,

1959; Razzell & Khorana, 1959)]. These alterations in the protein molecule are sufficient to allow separation of the different molecular species on the highly selective adsorbents now available. Autodigestion of the enzymes in solution is responsible for the appearance of new active components with papain and phosphodiesterase, and the hydrolytic action of resins themselves cannot be neglected as a possible contributing factor where strong adsorption occurs (Boardman, 1959).

Finally, it is possible that part of a protein component is associated with other material in the crude mixture and that this may be responsible for the appearance of several components. Thus Hjertén (1959) has attempted to explain the chromatographic heterogeneity of serum albumin on the basis of its capacity to bind small molecules, and Åquist & Anfinsen (1959) have suggested that the binding of polyanions may be responsible for the chromatographic differences among the various sheep ribonucleases.

It is possible that one or more of the factors listed here may explain the appearance of the chromatographically different forms of the β-glucosidases of *A. niger* and may also provide an explanation for the change in the numbers of components on rechromatography of the separated fractions.

SUMMARY

1. The β-glucosidases in an enzyme preparation from *Aspergillus niger* have been fractionated by gradient-elution chromatography on calcium phosphate and the anion-exchange resin Dowex-1. The activity of the fractions was tested against cellosaccharides (cellobiose, cellopentaose), cellulosic substrates (cellodextrin, sodium carboxymethylcellulose, phosphoric acid-swollen cellulose), laminarin, lichenin and salicin.

2. The activity towards the β-1:4-glucosidic substrates was obtained in two well-separated groups of components on both calcium phosphate and Dowex-1. The differences between these two groups of components are discussed.

3. The presence of β-glucosidase components hydrolysing laminarin but not cellulosic substrates was indicated by the results of Dowex-1 and calcium phosphate chromatography. From each adsorbent some fractions hydrolysing laminarin also hydrolysed cellosaccharides. On calcium phosphate those fractions with both activities were completely resolved from the remainder of the fractions hydrolysing laminarin.

4. Lichenin was hydrolysed chiefly by those fractions from Dowex-1 which hydrolysed cellulosic substrates.

5. Salicin was hydrolysed only by those fractions from Dowex-1 which hydrolysed cellosaccharides, but not all such fractions hydrolysed salicin.

6. For each of the β-glucosidases the activity is distributed among a number of components. The possible nature of these components is discussed.

The authors owe much to Dr E. M. Crook for his interest and advice. They also wish to thank Mr T. Guyver for technical assistance, Rohm and Haas Co. for a generous supply of *Aspergillus niger* enzyme, Dr D. R. Whitaker for supplying the cellopentaose and Dr J. A. Gascoigne for the viscosity determinations.

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Biochem. J. (1961) **78**, 723

The Determination of Haptoglobins in Normal Human Serum

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(*Received* 17 *May* 1960)

Haptoglobins are proteins which combine with haemoglobin or certain derivatives of haemoglobin. Under certain conditions, combination of haptoglobin with haemoglobin or methaemoglobin enhances the peroxidase activities of the latter substances (Polonovski & Jayle, 1940; Guinaud, Tonnelat, Boussier & Jayle, 1956). This finding has been made the basis of methods for the determination of haptoglobins. Jayle (1951) estimated haptoglobins in serum by measuring the effect of the haemoglobin–haptoglobin complex on the reaction between ethyl hydroperoxide and iodide ions; liberated iodine was determined by titration with thiosulphate. More recently Connell & Smithies (1959) have utilized the reaction between guaiacol and hydrogen peroxide to determine the peroxidase activity of methaemoglobin–haptoglobin complex, measuring the rate of oxidation of guaiacol colorimetrically.

* Working with the aid of a grant from the National Health and Medical Research Council of Australia.

This paper presents observations on the use of the guaiacol–hydrogen peroxide reaction in the determination of haptoglobins and on the concentration of haptoglobins in normal human serum. A preliminary account of certain aspects of the work has already appeared (Owen, Better & Hoban, 1960).

MATERIALS

Guaiacol reagent. Standard reagent (Connell & Smithies, 1959) was prepared as described by Owen, Better & Hoban (1960). In experiments to test the effect of pH, similar reagents were prepared in which the guaiacol concentration was kept constant (3·72 g./l.) and the pH varied with appropriate additions of 0·1 M-sodium acetate (pH 3·2–5·0) or sodium phosphate (pH 6·0–7·0) buffers.

Hydrogen peroxide. This was 0·05 M as described by Owen *et al.* (1960).

Methaemoglobin solution. To 10 vol. of human erythrocytes, previously washed three times with 0·15 M-NaCl, were added 10 vol. of water and 1 vol. of peroxide-free ether. The mixture was shaken for 1 min. and centrifuged,

and the clear haemolysate was pipetted off. The haemoglobin in the haemolysate was determined colorimetrically as oxyhaemoglobin with a calibration curve constructed from a standard haemoglobin solution supplied by Red Cross Transfusion Service, Sydney. The haemolysate was then diluted until the haemoglobin concentration was 1 g./100 ml. To 25 ml. of this solution was added 10 ml. of potassium ferricyanide (100 mg./100 ml.) to convert the haemoglobin into methaemoglobin. After 10 min. the volume was made up to 450 ml. The concentration of methaemoglobin was then determined by measurement of the extinction of the solution in a spectrophotometer (Unicam SP. 400) at 500 mμ, with a millimolar extinction coefficient (ε mM) of 38·2 (Allison & ap Rees, 1957). This coefficient is based upon haemoglobin; not upon haematin. The solution was then diluted until the concentration was exactly 50 mg./100 ml.

Sera. Normal blood samples were obtained from blood donors of European (Caucasian) descent. The samples were allowed to clot and serum was obtained in the usual way. Sera containing visible amounts of haemoglobin were discarded. Sera were normally diluted 1 in 5 with 0·15M-NaCl before assay.

METHODS

Assay of peroxidase activity. Unless otherwise stated, the calorimetric assay of haptoglobin was carried out as follows 1: ml. of the diluted serum was mixed with 1 ml. of methaemoglobin solution; 5·0 ml. of guaiacol reagent was then placed in a test tube in a water bath at 25°. After 10 min., 0·1 ml. of the mixture of test solution and methaemoglobin was added. Immediately after, 1·0 ml. of 0·05M-hydrogen peroxide, previously warmed to 25°, was added and the contents of the tube were mixed. After 10 min. the tube was removed from the bath and the extinction of the solution measured in a spectrophotometer (Unicam SP. 1400) at 470 mμ. With 10 mm. diameter matched test tubes the instrument was set to zero with water and readings were made within 1 min. With multiple tubes, timing was such that all tubes were treated similarly. A blank was performed with a mixture of equal volumes of test material and water. A calibration curve was prepared as described by Owen *et al.* (1960). Haptoglobin concentrations have been expressed in terms of bound methaemoglobin.

Electrophoretic analyses. Paper electrophoresis was carried out as described by Owen, Mackay & Got (1959). Starch-gel electrophoresis was carried out essentially as described by Smithies (1955). In determination of the haptoglobin type, haemoglobin was added to sera to give a final concentration of approx. 200 mg./100 ml. A dianisidine reagent (Owen, Silberman & Got, 1958) was used to locate zones containing the haemoglobin–haptoglobin complex after electrophoresis on paper or in starch gel.

RESULTS

Guaiacol reaction

The progress of the reaction under standard conditions and the effect of temperature are shown in Fig. 1. With the standard procedure and with diluted serum as a source of haptoglobin, the extinction of the reaction mixture reached a maximum at 10 min. and then decreased. At a higher temperature (30°) a lower peak value was reached sooner and was sustained for a shorter period. At a lower temperature (20°) a higher peak value was reached later and was sustained for longer. Fading of the developed colour was accelerated if the tubes were exposed to diffuse bright daylight, but it was not abolished by placing the tubes in the dark.

The effect of adding more of the methaemoglobin–haptoglobin complex to the reaction mixture after the peak extinction had been reached is shown in Fig. 2. In this experiment, an additional amount of the complex equal to that present initially was added. The increment was considerably higher than the first peak.

The increase in sensitivity of the guaiacol reagent on aging is demonstrated in Fig. 3. The reagent was stored at room temperature (about 20°) in diffuse daylight. Storage at 2° decreased the aging effect but did not abolish it.

The effect of pH on the reaction is shown in Fig. 4. The optimum pH for colour development with the methaemoglobin–haptoglobin complex was about 4 whereas the optimum pH for colour development with methaemoglobin was about 6.

The method of using pooled normal serum to construct a calibration curve has been described by

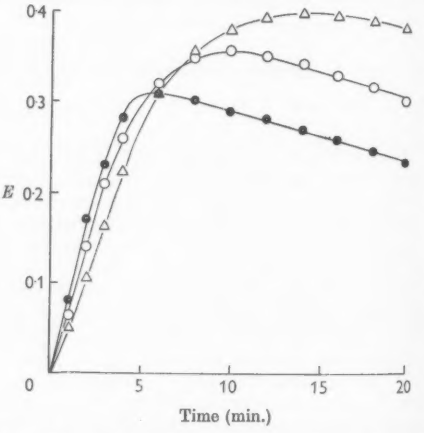

Fig. 1. Effect of temperature on the reaction between guaiacol and hydrogen peroxide in the presence of methaemoglobin–haptoglobin complex. The standard procedure (see text) was used except that readings were made at intervals and various temperatures were employed. Serum contained 95 mg. of haptoglobin/100 ml. Colours were developed in the photometer cuvettes, which were removed from the water bath for 10 sec. for each reading. Room temperature was 23·5°. △, 20°; ○, 25°; ●, 30°.

Owen *et al.* (1960), who give a typical curve. The curve covers the range 0–50 mg./100 ml. Usually sera were diluted 1 in 5 so that the range covered with sera was 0–250 mg./100 ml. If sera gave values within 10% of the peak value a fresh assay was set up by using a higher dilution. When sera diluted 1 in 5 gave values (uncorrected for dilution) below 10 mg./100 ml. they were reassayed without dilution. This uncovered an anomalous effect. Apparent haptoglobin values for such sera varied with the dilution (Table 1). Paper electrophoresis of these sera gave values in agreement with those obtained by the colorimetric procedure without dilution of serum. The dilution effect was only noticeable when sera with low haptoglobin contents were diluted before assay. No dilution effect was noticed when sera with high (i.e. > 40 mg./100 ml.) haptoglobin contents were diluted.

The reproducibility of the method was examined by assaying replicate samples of two sera. The results for the mean ± standard deviation and coefficient of variation respectively were: serum A (n 6) 145 ± 3·6, 2·5%; serum B (n 12) 197 ± 4·1, 2·1%.

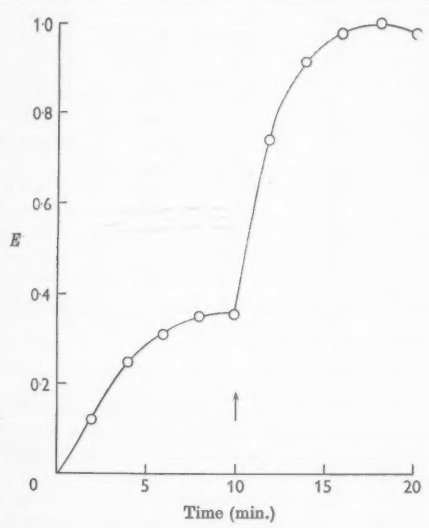

Fig. 2. Destruction of the peroxidase activity of methaemoglobin–haptoglobin complex in the presence of guaiacol and hydrogen peroxide. Standard procedure (see text) was used. Serum contained 95 mg. of haptoglobin/100 ml. Colour was developed in the photometer cuvettes which were removed from the water bath for 10 sec. for each reading. Room temperature was 23·5°. At the time indicated by the arrow a quantity of methaemoglobin–haptoglobin complex, equal to that present initially, was added.

Haptoglobins in normal human serum

Sera were obtained from 152 Caucasiun blood donors and the haptoglobin contents determined by the standard procedure. The genetic haptoglobin type was determined by starch-gel electro-

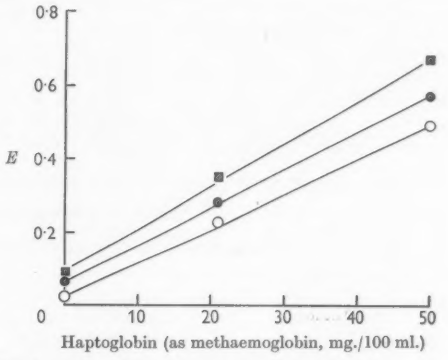

Fig. 3. Effect of age of guaiacol reagent on the reaction between guaiacol and hydrogen peroxide in the presence of methaemoglobin and of methaemoglobin–haptoglobin complex. Standard procedure (see text) was used. The guaiacol reagent had been stored at room temperature (about 20°) for: ○, 1 day; ●, 12 days; ■, 20 days. In each case the reaction was carried out with methaemoglobin alone (haptoglobin, absent), with a mixture of mathaemoglobin and methaemoglobin–haptoglobin complex (haptoglobin, 21 mg./100 ml.) and with methaemoglobin–haptoglobin complex alone (haptoglobin, 50 mg./100 ml.).

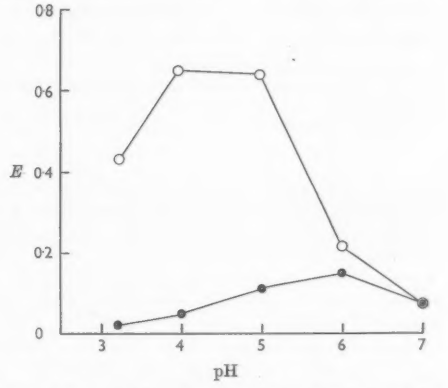

Fig. 4. Effect of pH on the reaction between guaiacol and hydrogen peroxide in the presence of methaemaglobin (●) and methaemoglobin–haptoglobin complex (○) respectively. Serum contained 95 mg. of haptoglobin/100 ml. Standard procedure was used except that serum was not diluted, i.e. all methaemoglobin was bound to haptoglobin.

Table 1. *Effect of dilution on the apparent haptoglobin content of sera with low haptoglobin contents*

Haptoglobin concentrations are expressed in terms of bound methaemoglobin. Serum A, normal; serum B, from patient with pernicious anaemia. The standard procedure (see text) was employed except that various dilutions of serum were assayed.

	Serum A		Serum B	
Dilution factor	$E_{470\ m\mu}$	Apparent haptoglobin concn. (mg./100 ml.)	$E_{470\ m\mu}$	Apparent haptoglobin concn. (mg./100 ml.)
1	0·087	4	0·143	9
5	0·083	20	0·094	23
10	0·074	32	0·086	37
20	0·065	42	0·074	54
(Blank)	0·045	—	0·051	—

Table 2. *Haptoglobins in normal human serum*

Results are expressed as mg. of bound haemoglobin or methaemoglobin/100 ml.

Author	Method	*n*	Haptoglobin type*	Haptoglobin concn. Mean	S.D.	Actual range
Present	Peroxidase–guaiacol	152‡	All	93	40	4–220
		21	1·1	104	34	21–155
		80	2·1	102	37	28–220
		49	2·2	72	38	14–193
Nyman (1959)	Peroxidase–iodide (Jayle, 1951)	277	All	110	41	20–250
		58	1·1	136	30	50–220
		130	2·1	108	37	30–220
		86	2·2	82	34	20–180
Lathem & Worley (1959)	Paper electrophoresis	10	All	128	—	98–171
Lathem & Jensen (1959)	Paper electrophoresis	28	All	102	30	68–170
Owen, Mackay & Got (1959)	Paper electrophoresis	50	All	84	—	30–200
Allison (1958)	Starch-gel electrophoresis	—	1·1	120	26	—
		—	2·1	120	26	—
		—	2·2	100	20	—
Jayle & Vallin (1952)	Peroxidase–iodide	1000	All	74†	31	—
Brissaud (1941)	Peroxidase–iodide	—	All	69†	—	26–111

* Nomenclature of Smithies (1955).
† On the assumption that 1 Haptoglobin Index unit corresponds to a haemoglobin-binding capacity of 85 mg./100 ml. (Jayle, 1951).
‡ Includes two sera in which very low haptoglobin concentrations precluded typing.

phoresis. The results are shown in Table 2. The haptoglobin contents of the sera ranged from 4 to 220 mg./100 ml. Two sera contained so little haptoglobin (4 and 11 mg./100 ml.) that it was not possible to determine the haptoglobin type.

Sera from a further 800 Caucasian donors were examined qualitatively for the presence of haptoglobin by means of paper electrophoresis. Haemoglobin was added to the sera before electrophoresis to give a concentration of 200 mg./100 ml. of serum and the stained electrophoretic strips were examined for peroxidase activity in the α_2-globulin zone. All sera showed some peroxidase activity in this position, though in some the activity was slight. In some sera, a peroxidase area was also noted in the albumin zone. This was presumably due to the presence of methaemalbumin and was found whenever sera were kept with haemoglobin in excess of the haptoglobin–haemoglobin-binding capacity. Thus methaemalbumin was detected in sera which contained excess of haemoglobin and which had stood at 2° for 12 hr. or at room temperature for shorter periods.

DISCUSSION

The complex nature of the overall reaction between guaiacol, hydrogen peroxide and the methaemoglobin–haptoglobin complex is illustrated in Figs. 1–3. The cessation of colour de-

velopment within minutes is presumably due to destruction of the peroxidase activity of the methaemoglobin–haptoglobin complex, for addition of more complex allows the reaction to proceed again for a while. Inactivation of other peroxidases by hydrogen peroxide is well recognized (Lemberg & Legge, 1949).

The immediate oxidation product of guaiacol is tetraguaiacol. However, this substance is not stable (Maehly, 1954). Reference to Fig. 1 indicates that fading is less at 20° than at higher temperatures, which suggests that this temperature (20°), or lower, would be more suitable for the measurement of haptoglobins. However, in Melbourne the room temperature is frequently higher than 20° and, to avoid the need for a water bath maintained below room temperature, a temperature of 25° was chosen for the standard procedure.

The increased sensitivity of the guaiacol reagent on aging or after some colour has been developed are possibly different expressions of the same phenomenon. Certainly aged guaiacol develops a slight brown tinge. It seems possible that an oxidation product of guaiacol, or of some impurity present, increases the rate of oxidation of guaiacol by hydrogen peroxide under the influence of peroxidase. Whatever the nature of the phenomenon allowance has to be made for it, and for precise work a calibration curve should be constructed each time the method is used.

We cannot at present offer a certain explanation for the dilution anomaly noted with sera containing very low haptoglobin levels though it may possibly be due to the presence of a β-globulin with weak haemoglobin-binding capacity (Neale, Aber & Northam, 1958; Nyman, 1959). We believe, however, that the values obtained for such sera by the colorimetric procedure without prior dilution of the serum are correct, since they agree with the results obtained by the electrophoretic method. Accordingly it is our practice to assay sera diluted initially 1 in 5. Any serum which has an apparent haptoglobin content of less than 50 mg./100 ml. is then reassayed without dilution.

The method described here for the estimation of haptoglobins is simpler than that devised by Connell & Smithies (1959), which requires a temperature-controlled spectrophotometer. Hommes (1959) has described a method involving isolation of the haemoglobin–haptoglobin complex by paper electrophoresis and estimation of its peroxidase activity by a relatively simple method. However, the electrophoresis requires several hours as does the electrophoresis method of Lathem & Worley (1959). Jayle's (1951) method is simple and precise but involves the use of ethyl hydroperoxide, a potentially dangerous reagent.

The coefficient of variation of results obtained with the present method is slightly less than that found by Nyman (1959) using the method of Jayle (1951), i.e. 2·6.

The present method gives values for haptoglobin essentially in agreement with those obtained by other methods (Table 2). Moreover, our findings confirm earlier reports (Allison, 1958; Nyman, 1959) that the serum haptoglobin level, in terms of haemoglobin-binding capacity, depends on the genetic haptoglobin type. The dependence of the mean haemoglobin-binding capacity of sera on the haptoglobin type could be due to differences in the haemoglobin-binding capacity (mg./mg.) of the different molecular species of haptoglobin or to different amounts of haptoglobin. It has been suggested (Allison, 1959) that haptoglobin type 1·1 consists of monomeric forms whereas haptoglobin type 2·2 consists mostly of polymeric forms. If this is correct it is conceivable that, with type 2·2 haptoglobin, steric hindrance would decrease the haemoglobin-binding capacity relative to that of type 1·1 haptoglobin.

Our failure to find any normal serum completely lacking in haptoglobin is of some interest in view of the reports (Sutton, Neel, Binson & Zuelzer, 1956; Allison, Blumberg & ap Rees, 1958; Harris, Robson & Siniscalo, 1958; Kirk, Lai & Hogben, 1960) that a small proportion of Caucasians do not have any serum haptoglobin. However, these authors all used starch-gel electrophoresis whereas in our experience starch-gel electrophoresis, with the filter-paper method of sample insertion (sample volume, 0·02 ml.), constitutes a less sensitive method of detecting haptoglobin than paper electrophoresis. Thus in our hands with (pathological) sera containing less than 10 mg. of haptoglobin/100 ml. and with added haemoglobin it is usually not possible to demonstrate haptoglobin (peroxidase-active) zones after electrophoresis in starch gel, although haptoglobin in concentrations as low as 2 mg./100 ml. is easily detected on paper electrophoresis. Our findings, therefore, together with those listed in Table 2 and other data (Galatius-Jensen, 1958; Moullec & Fine, 1959), indicate that complete ahaptoglobinaemia is rare in Caucasians if indeed it occurs at all.

Assuming that the serum haptoglobin types 1·1 and 2·2 represent homozygous alleles and that type 2·1 is a heterozygous form (Smithies & Walker, 1955; Kirk *et al.* 1960) the gene frequencies in our normal material are: Hp 1 gene 40·7 %, Hp 2 gene 59·3 %. In 150 persons this would give an expected approximate distribution of haptoglobin types as follows: type 1·1, 24; type 2·1, 80; and type 2·2, 54. These values do not differ significantly (χ^2, 1·6; $P > 0·5$) from the observed values (Table 2). The greater incidence of the Hp 2 gene in

Caucasians has been reported previously (for references, see Kirk *et al.* 1960).

Note added in proof. Since submitting this paper, we have observed that both caeruloplasmin and a globulin–haem complex (see Nyman, 1960) react with the dianisidine reagent used to detect the haptoglobin–haemoglobin complex after paper electrophoresis. Although caeruloplasmin migrates clearly ahead of the haptoglobin–haemoglobin complex on paper electrophoresis at pH 6·5, the globulin–haem complex and the haptoglobin–haemoglobin complex do not separate under these conditions. Accordingly the finding of a dianisidine reactive area in the α_2-globulin zone (pH 6·5) does not necessarily indicate the presence of the haptoglobin–haemoglobin complex. Since some of the 800 normal Caucasian sera examined by paper electrophoresis were found to contain methaemalbumin, it is possible that they also contained the globulin–haem complex. Consequently our findings do not necessarily exclude a haptoglobinaemia in these sera.

SUMMARY

1. The peroxidase activities of methaemoglobin and of methaemoglobin–haptoglobin complex on the reaction between guaiacol and hydrogen peroxide have been examined and a method for the determination of haptoglobins in serum was devised.

2. The method has been used to determine the serum haptoglobin concentration in normal human serum. A mean value (as bound methaemglobin) of 93 mg./100 ml. (S.D. 40) was obtained. The mean serum-haptoglobin concentration varied according to the genetic haptoglobin type.

We should like to thank the Blood Bank, St Vincent's Hospital, Melbourne, for providing the blood samples. Miss A. Jowett and Mr R. Padanayi provided valuable technical assistance.

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The Glycolytic Enzymes of Guinea-Pig Lung in Experimental Bagassosis

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(*Received* 30 *May* 1960)

In a previous study of experimental bagassosis Singh, Mitra, Venkitasubramanian & Viswanathan (1960) showed that in this condition there was an appreciable change in the activity of various respiratory enzymes and in the Q_{O_2} of guinea-pig lung. Also, V. N. Singh, T. A. Venkitasubramanian & R. Viswanathan (unpublished work) found a remarkable alteration in the chemical composition of

lung. These results suggest that there may be a metabolic derangement in guinea pigs exposed to bagasse dust. The latter study demonstrated, together with other alterations, an increase in the glycogen content of guinea-pig lung as a result of experimental bagassosis.

It might be expected that a study of the enzyme systems involved in the synthesis and breakdown of glycogen would contribute to an understanding of the mechanism of metabolic response of guinea pigs to experimental bagassosis. To obtain an insight into the mechanism of glycogen accumulation a study of certain important enzymes of the glycolytic pathway, namely phosphorylase, hexokinase, phosphoglucoisomerase and aldolase, as well as the estimation of lactic acid in lung tissue of guinea pigs exposed to bagasse dust, has been made.

MATERIALS AND METHODS

Chemicals. Glucose 1-phosphate, adenosine triphosphate, glucose 6-phosphate and fructose-1:6-diphosphate were all Sigma Chemical Co. products.

Fructose 1:6-diphosphate as the barium salt was dissolved in N-HCl and freed from barium by addition of the required amount of Na_2SO_4. The slight yellow colour of the solution was removed by shaking with Norit charcoal. The solution was adjusted to pH 8·6 by the addition of NaOH, filtered, and the concentration of fructose diphosphate in the solution was determined by estimating fructose by the method of Roe (1934). The solution was then diluted to give a concentration of 0·05 M.

Lactic acid (85%) was diluted to approx. 1·0 M, slightly over-neutralized with NaOH, boiled, and then back-titrated and diluted appropriately.

Animals. Young guinea pigs (300–500 g.) were selected and paired for weight, age and sex. They were fed without restraint. One guinea pig of each pair was kept as a control, and the other one was exposed to bagasse dust by a mechanical device (Singh *et al.* 1960) for various periods of time as described in the Results section.

Tissue collection. Animals were stunned by a blow on the head and decapitated. Lung tissue was removed as rapidly as possible, freed from adhering blood with filter paper and kept in a container cooled in an ice-salt mixture. Lung tissue was used for the various estimations as quickly as possible.

Enzyme assays

Phosphorylase. The methods of Shull, Ashmore & Mayer (1956) and of Cori, Cori & Green (1943) were modified to make the procedure applicable to lung. The lung tissue was homogenized in 0·1 M-NaF to give a 15% (w/v) homogenate. Fluoride served the purpose of inactivating phosphoglucomutase (Najjar, 1948), which would otherwise interfere with the estimation by converting glucose 1-phosphate into glucose 6-phosphate. Suitable amounts of homogenate were pipetted into graduated centrifuge tubes containing 0·7 ml. of 0·154 M-NaF, 0·2 ml. of 0·25 M-glucose 1-phosphate, 0·6 ml. of 0·1 M-citrate buffer (pH 5·8), 0·1 ml. of 4% (w/v) glycogen, and the volume was made up to 2·0 ml. with water. The reaction mixture was incubated for 30 min. at 30° and the reaction was then stopped by the addition of 1·0 ml. of ice-cold 10% (w/v) trichloroacetic acid. The volume was made up to 10·0 ml. and after the tubes were kept in an ice bath for 20 min. they were centrifuged, and the solution was filtered through Whatman no. 42 filter paper. The filtrate was taken for the estimation of inorganic phosphorus by the method of Sumner (1944) with slight modifications. To 2 ml. of the filtrate, taken in a test tube and made up to 8·2 ml. with water, 0·5 ml. of 6·6% (w/v) ammonium molybdate, 0·5 ml. of 7·5 N-H_2SO_4 and 0·8 ml. of freshly prepared 10% (w/v) $FeSO_4$ (reducing agent) were added. Standard phosphorus (15, 30 and 45 μg.) controls were also run in each experiment. The extinction was read after 10 min. at 660 mμ in a Bausch and Lomb colorimeter.

For each concentration of homogenate a blank was prepared with the enzyme inactivated at zero time.

Hexokinase. Activity was estimated by the method of Long (1952) with 25% (w/v) lung homogenate. Because of the inhibitory action of Na^+ ions on hexokinase activity (Wiebelhaus & Lardy, 1949) an all-potassium medium as recommended by Long (1952) was used. The incubation period was 10 min. Glucose was estimated by the Somogyi (1952) method as recommended by Kerly & Leaback (1957). For each concentration of the homogenate a separate blank was run with the enzyme inactivated at zero time. For every experiment a reference standard curve of glucose was prepared.

Phosphoglucoisomerase. The method of Glock, McLean & Whitehead (1956) based on that of Slein (1955) was followed for the assay of the enzyme with 0·2% (w/v) tissue homogenate. The fructose 6-phosphate formed was determined colorimetrically by the method of Roe (1934), the extinction being read at 490 mμ in a Bausch and Lomb colorimeter. Experimental details were those described by Glock *et al.* (1956) except that 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·6, replaced glycylglycine buffer. For each concentration of homogenate a blank was run where enzyme activity was stopped at zero time. The concentration of fructose 6-phosphate was determined by referring to a standard fructose solution which was analysed with every experiment.

Aldolase. Activity of this enzyme was estimated by the method of Sibley & Lehninger (1949) by using 2% (w/v) tissue homogenate. The homogenate was brought to 38° before addition to the reaction mixture, as suggested by Swenson & Boyer (1957). The enzyme was inactivated at the end of the incubation period of 15 min. by addition of 10% (w/v) trichloroacetic acid as recommended by Beck (1955), and the tubes were kept in ice for 10 min. (Roodyn, 1956). After addition of 2:4-dinitrophenylhydrazine the tubes were incubated at 38° for 30 min. (Beck, 1955) for colour development.

For each homogenate concentration a blank was prepared in which the homogenate was added only after the addition of trichloroacetic acid. The extinction was read at 540 mμ, and results were calculated by referring to a standard curve (Sibley & Lehninger, 1949).

Determination of lactic acid. The method of Barker & Summerson (1941) was followed. As soon as the animal was dissected a small portion of the lung tissue was freed from adhering blood with filter paper and transferred to weighed 15 ml. centrifuge tubes containing 2 ml. of 10% (w/v) trichloroacetic acid. The tubes were reweighed to obtain the weight of the tissue. Trichloroacetic acid was added to

Table 1. *Effect of experimental bagassosis on phosphorylase, phosphoglucoisomerase, hexokinase and aldolase activity of guinea-pig lung*

Details of the assay procedures are given in the Methods section. Values are given as means ±S.E. (with number of experiments shown in parentheses).

Time of exposure to dust (hr.)	(*a*) Phosphorylase: Inorganic phosphorus liberated (μg. atoms/g. wet wt./min.)	(μg. atoms/g. dry wt./min.)
Nil	2·59±0·06 (9)	12·23±0·52 (9)
44–114	2·46±0·07 (9)	12·81±0·58 (9)
	$0{\cdot}2 > P > 0{\cdot}1$	$0{\cdot}5 > P > 0{\cdot}4$
	(*b*) Phosphoglucoisomerase: Fructose 6-phosphate liberated	
	(μmoles/g. wet wt./min.)	(μmoles/g. dry wt./min.)
Nil	90·8±1·6 (6)	429·8±12·1 (6)
51–68	133·8±2·1 (6)	682·5±14·6 (6)
	$P < 0{\cdot}001$	$P < 0{\cdot}001$
	(*c*) Hexokinase: Glucose used	
	(μmoles/g. wet wt./min.)	(μmoles/g. dry wt./min.)
Nil	0·33±0·03 (7)	1·38±0·07 (7)
51–63	0·67±0·06 (7)	3·81±0·64 (7)
	$P < 0{\cdot}001$	$P < 0{\cdot}005$
	(*d*) Aldolase: Hexose diphosphate used	
	(μmoles/g. wet wt./min.)	(μmoles/g. dry wt./min.)
Nil	3·26±0·07 (8)	12·83±0·26 (8)
52–63	3·94±0·22 (8)	18·37±1·01 (8)
	$0{\cdot}02 > P > 0{\cdot}01$	$P < 0{\cdot}001$

minimize the error due to glycolysis. The tissue was homogenized and diluted to give a concentration of 0·4% (w/v) of homogenate. A reagent blank was run through the complete procedure and a standard curve for lactic acid was prepared every time.

RESULTS

Results of the enzyme assays are given in Table 1. In studying phosphorylase activity, experimental animals were exposed to the bagasse dust for periods varying from 44 to 114 hr. Nine animals from each group, control and experimental, were used for the assay. As can be seen from the Table, experimental bagassosis does not induce any significant change in the phosphorylase activity of guinea-pig lung, although the trend, if any, seems to be towards a decrease.

Hexokinase activity is reported in the same Table. Experimental animals used for the assay of this enzyme were exposed to bagasse dust for periods varying from 51 to 63 hr. It is evident that hexokinase activity of guinea-pig lung is significantly increased in experimental bagassosis.

Results of the estimation of phosphoglucoisomerase show that the activity of this enzyme in guinea-pig lung was remarkably enhanced in bagassosis.

Aldolase activity is also given in Table 1. It is evident that aldolase activity of guinea-pig lung is significantly increased in bagassosis.

Results on the lactic acid content of lung are given in Table 2. The results are expressed in terms of (i) μmoles of lactic acid/g. of wet tissue, (ii) lactic acid (μmoles) of whole lung tissue/100 g. body wt. and (iii) total lactic acid (μmoles) in the whole lung, which will be referred to as total lactic acid. As can be seen from the Table, the lactic acid content of guinea-pig lung was significantly increased in bagassosis.

DISCUSSION

As the moisture content of guinea-pig lung is increased by exposure to bagasse dust, it was thought desirable to express the results both as per g. of wet tissue and per g. of dry tissue. Conversion of the results on wet tissue basis to those on dry

Table 2. *Lactic acid content of lung tissue of normal guinea pigs and those exposed to bagasse dust*

For experimental details see the Methods section. Figures of results are means ±S.E. with number of experiments shown in parentheses.

	Normal guinea pigs	Experimental guinea pigs (exposed to dust 41–115 hr.)	Significance of difference
Lactic acid (μmoles/g. of wet tissue)	10·9±0·72 (9)	14·7±1·3 (9)	$0·02 < P < 0·025$
Lactic acid (μmoles/100 g. body wt.)	8·8±0·46 (9)	16·7±2·0 (9)	$0·001 < P < 0·005$
Total lactic acid (μmoles/whole lung)	38·3±2·8 (9)	70·3±9·2 (9)	$0·001 < P < 0·005$

tissue basis appears to give a different ratio for normal tissue on one hand and experimental tissue on the other. This can be explained by the increased moisture content of guinea-pig-lung tissue in experimental bagassosis.

The discovery of Leloir & Cardini (1957) of an enzyme in liver which brings about uridine diphosphate glucose-linked synthesis of glycogen and the subsequent studies of this enzyme in several other tissues (Leloir, Olavarria, Goldemberg & Carminatti, 1959; Villar-Palasi & Larner, 1960) have stimulated further extensive studies in this field. Considerable evidence has recently accumulated suggesting that under physiological conditions the uridine diphosphate glucose-linked pathway serves an anabolic function whereas phosphorylase plays a catabolic role in glycogen metabolism (Sutherland, 1956; Robbins & Lipmann, 1959; Schmid & Mahler, 1959; Schmid, Robbins & Traut, 1959; Larner & Villar-Palasi, 1959). Thus an almost unaltered phosphorylase activity of guinea-pig lung in experimental bagassosis suggests that there is no appreciable change in the catabolic phase of glycogen metabolism.

When the role of hexokinase in diseases of carbohydrate metabolism such as diabetes (Price, Cori & Colowick, 1945; Cori, 1945–46; Hoffman, 1949; Chernick, Chaikoff & Abraham, 1951) is considered, the increase in the activity of this enzyme in guinea-pig lung in experimental bagassosis is of interest. Increased hexokinase activity will augment the phosphorylation of glucose and thereby increase the glucose 6-phosphate pool of the diseased guinea-pig lung. The increased glucose 6-phosphate could be channelled towards the synthesis of glycogen as well as towards the glycolytic pathway which leads finally to the production of lactic acid or pyruvic acid, depending on the supply of oxygen.

The activities of phosphoglucoisomerase and aldolase of guinea-pig lung are also increased in experimental bagassosis and thus the rates of the following reactions will be accelerated:

glucose 6-phosphate ⇌ fructose 6-phosphate

and

fructose 1:6-diphosphate ⇌ triose phosphates.

At present it is not possible to decide whether the increase in these two enzymes is a primary effect of the disease or a secondary effect of the increased glucose 6-phosphate pool.

The elevated lactic acid content of guinea-pig-lung tissue in experimental bagassosis is also of interest. Hypoxia, which is induced by experimental bagassosis in guinea-pig lungs, would lead to the predominance of anaerobic glycolysis in order to cope with the demand for oxidized pyridine nucleotides and with the requirement of energy, thereby augmenting the production of lactic acid.

The enhanced hexokinase, phosphoglucoisomerase and aldolase activities, as well as the unaltered phosphorylase activity, suggest that the increase in glycogen content observed by V. N. Singh, T. A. Venkitasubramanian & R. Viswanathan (unpublished work) is due to increased glycogen synthesis via the uridine diphosphate glucose-linked route without any alteration in its catabolic phase. It seems reasonable to assume here that increased hexokinase activity augments the phosphorylation of glucose and that the glucose 6-phosphate thus produced is channelled both towards the synthesis of glycogen, producing thereby an increase in the glycogen content, and towards the glycolytic pathway, thereby increasing the production of lactic acid in guinea-pig lung in experimental bagassosis.

SUMMARY

1. The activity of certain glycolytic enzymes, namely phosphorylase, hexokinase, phosphoglucoisomerase and aldolase, was estimated in lungs of guinea pigs which have been exposed to bagasse dust and was compared with that of normal guinea-pig lungs.

2. Phosphorylase activity was unaltered, whereas the activities of the other three enzymes were significantly increased in experimental bagassosis.

3. The lactic acid content was increased in the lungs of guinea pigs exposed to bagasse dust as compared with those of normal ones.

4. The significance of the results in explaining the increased glycogen content of guinea-pig lung in experimental bagassosis is discussed.

Our grateful thanks are due to the Indian Council of Medical Research, New Delhi, for financial grants.

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Biochem. J. (1961) **78**, 732

Distribution of Unsaturated Fatty Acid in Pyridoxine-Deficient Hypercholesterolaemia

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(*Received* 30 *June* 1960)

The present study was prompted by the suggestion of Sinclair (1956) that the development of atherosclerosis may be related to a deficiency of unsaturated fatty acids. Since pyridoxine is known to be a factor for the conversion of linoleate into arachidonate (Witten & Holman, 1952), the atherosclerosis observed in pyridoxine-deficient monkeys (Rinehart & Greenberg, 1959) may be related to the deficiency of arachidonate. To test the validity of this hypothesis, a detailed study has been made of the unsaturated fatty acid content of the blood and arteries of pyridoxine-deficient rats.

Whether the hypercholesterolaemia observed was due to increased mobilization of lipids from liver or to some intrinsic metabolic aberration was tested by determining the cholesterol content of bile, which is known to reflect faithfully the status of cholesterol synthesis by the liver (Friedman & Byers, 1954).

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EXPERIMENTAL

Induction of pyridoxine deficiency. Rats (60) of 60–85 g. body wt. were fed on a purified diet containing vitamin-free casein (22·0%), sucrose (66·5%), fat (coco-nut oil; 9·0%), salt mixture (2·5%) and sulphaguanidine for depletion of vitamins. After 3 weeks the animals were divided into three groups of 20 each. Animals of group 1 served as control and received the basal diet supplemented with a vitamin mixture containing (mg./kg. of diet) the following: calcium pantothenate (44); vitamin A concentrate (200 units/ml.; 99); α-tocopherol (225·5); inositol (301·4); menadione (4·4); biotin (0·7); niacin (595·5); *p*-aminobenzoic acid (22); riboflavin (22); pyridoxine hydrochloride (46); thiamine hydrochloride (22); choline chloride (601·0); folic acid (0·2 μg./kg.). Group 2 animals (inanition group) also received the same diet but it was adjusted in amount so that their weight gain corresponded to that of group 3 rats (deficient group), which received the same diet with the exception of pyridoxine hydrochloride. Body-weight gains were recorded daily and after 9 weeks the animals were killed. Blood was drawn directly from the heart after

opening the thorax and serum obtained from two animals of the same group was pooled for subsequent analyses.

Determination of unsaturated fatty acid of serum. (*a*) Preparation of lipid extract. A portion (2 ml.) of serum was pipetted dropwise into 10 ml. of ethanol–ether mixture (3:1, v/v) and refluxed under nitrogen for 8 hr. Samples were filtered and the residue was further extracted with 200 ml. of ethyl ether for an additional 8 hr. under nitrogen. The two extracts were combined and dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure and the total lipids were re-extracted with light petroleum (b.p. 60–70°).

(*b*) Separation of lipids on silicic acid column. A column for operation under slight but constant pressure of nitrogen (2 cm. Hg above atmospheric pressure) according to the design of Herb, Witnauer & Riemenschneider (1951) was used. The glass column (21 mm. × 300 mm.) was packed by introducing silicic acid (E. Merck, analytical grade, suitable for chromatography), prepared by boiling 22 g. of silicic acid in 100 ml. of light petroleum (b.p. 45–60°) for 5 min., with slow stirring with a stainless-steel rod. The column, when settled, was about 19 cm. high. About 10 ml. of the lipid solution was then placed at the top of the column with a separating funnel. This solution was washed down the column with 10 ml. of the solvent. The first eluting solvent was then added at the top of the column and the flow rate adjusted at a rate of about 150 ml./hr. by increasing or decreasing the nitrogen pressure at the top of the column. The order of elution was (1) hydrocarbons: 50 ml. of light petroleum (+1% of ethyl ether); (2) sterol esters: 300 ml. of light petroleum (+1% of ethyl ether); (3) glycerides: 300 ml. of light petroleum (+4% of ethyl ether); (4) sterol and free acids: 350 ml. of light petroleum (+8% of ethyl ether); (5) phospholipids: 250 ml. of methanol–ethyl ether (25:75, v/v) followed by 250 ml. of methanol–ethyl ether (50:50, v/v) (Luddy, Barford & Riemenschneider, 1958).

The solvent was removed from the eluted fractions under reduced pressure and transferred to round-bottomed flasks with light petroleum. This solvent was then removed *in vacuo* and the residue saponified with ethanolic KOH. The fatty acids were then extracted quantitatively with light petroleum after acidification and made to standard volume with light petroleum.

(*c*) Isomerization. A portion (15 ml.) of the solution was then transferred to reaction tubes designed according to Pikaar & Nijhof (1958). The solvent was evaporated in a water bath under a flush of nitrogen. A portion (0·5 ml.) of the KOH–ethylene glycol reagent was added and the tube heated to 180° for exactly 16 min. The tubes were then cooled and the volume was made to 10 ml. with methanol. Extinction values were measured in a Unicam SP. 500 spectrophotometer at 233, 268, 315, 346 and 374 mμ, against a blank similarly treated. The concentrations of polyethenoid fatty acids were calculated by using the equations of Herb & Riemenschneider (1952).

In the determinations of unsaturated fatty acids, it has been found desirable not to warm the reagent before addition of the sample. Nevertheless, the application of the formulae of Herb & Riemenschneider (1952) remains justifiable, as pointed out by several observers (Pikaar & Nijhof, 1958).

Fatty acids of arterial tissue. These were determined by the method of Dam & Engel (1958). Samples (10 ml.) of the light petroleum extracts were evaporated in reaction tubes and isomerized with KOH–ethylene glycol followed by determinations of extraction in the u.v. region. The total fatty acid content was estimated by weighing the residue after evaporation and also by titrating a sample dissolved in benzene with 0·005N-sodium ethoxide with phenolphthalein as indicator.

Iodine value. Samples of a solution of weighed fatty acids were transferred to glass-stoppered 50 ml. flasks; the solvent was evaporated under a stream of nitrogen and the contents were redissolved in chloroform. Exactly 1·0 ml. of 0·2N-Wijs solution was added, and the flasks were stoppered and kept in the dark for 1 hr. After this reaction time 0·5 ml. of 15% KI was added, followed by 5 ml. of water. The iodine was then titrated with 0·01N-sodium thiosulphate with 4–8 drops of 1% starch as indicator.

Bile. This was collected from fistulae made on rats under urethane anaesthesia, and bile and serum cholesterol were determined by the method of Mookerjea & Sadhu (1955).

RESULTS

The fatty acid deposition of the animals in general appear to be a little low when compared with results obtained by others, which is probably due to the inclusion of coco-nut oil as the dietary source of fat. There was, however, no appreciable deficiency in respect of essential fatty acids of the animals, as judged morphologically, and the differences between the control and the experimental groups in respect of the polyenoic acid concentration of the sterol-ester fractions of blood serum are significant (Tables 1 and 2). That coco-nut oil itself has an insignificant effect on the cholesterol of the rat is borne out by the observations of Nath, Weiner, Harper & Elvehjem (1959). Blood cholesterol (free) was 43·0 ± 4·8 mg./100 ml. in controls and 80·0 ± 3·1 mg./100 ml. in the experimental animals, the corresponding values for the ester fraction being 62·0 ± 2·7 and 118·0 ± 5·3 mg./100 ml. The aortic arch and the thoracic aorta have cholesterol values of 210·0 ± 8·5 mg./100 g. in the controls and 325·0 ± 12·5 mg./100 g. in the deficient animals.

DISCUSSION

There is increased cholesterol deposition in the arteries of pyridoxine-deficient rats, accompanied by esterification of sterol esters of blood with more saturated fatty acids, supporting the thesis of Sinclair (1956). Whether the arterial-sterol deposition is due to esterification with more saturated fatty acids cannot be said definitely as yet. Pyridoxine deficiency affects arachidonic acid formation and if cholesterol deposition in aortae of pyridoxine-deficient rats is due to more saturated sterol esters, there may be less cholesterol arachidonate in the aorta wall. This, combined with the increased amount of saturated fatty acids deposited with cholesterol in the arterial wall, will be reflected in the total fatty acid spectrum of the aortae in such a way as to lower its iodine value and arachidonate

Table 1. *Polyenoic fatty acid composition of arterial tissue of control rats and rats made deficient in pyridoxine*

Analysis on fatty acids recovered after saponification and removal of unsaponifiable material. Total fatty acid is expressed as g. and cholesterol as mg./100 g. wet wt. of tissue. Results are means ±S.D. for 20 rats in each group.

Acid	Control group	Pyridoxine-deficient group
Iodine value	63·5	68·7
Total fatty acids	4·3 ±0·38	4·2 ± 0·73
Oleic acid (%)	40·01±3·86	44·67± 2·31
Dienoic acid (%)	20·09±1·31	18·38± 0·97
Trienoic acid (%)	2·38±0·38	3·10± 0·56
Tetraenoic acid (%)	9·87±0·96	10·13± 0·68
Pentaenoic acid (%)	1·26±0·93	0·99± 0·39
Hexaenoic acid (%)	Traces	Traces
Cholesterol, free	210·0 ±8·5	325·0 ±12·5

Table 2. *Polyenoic fatty acid composition of blood-serum sterol esters of control rats, inanition-group rats and rats made deficient in pyridoxine*

Analysis on fatty acids of sterol esters separated from a silicic acid column of Bloor extract. Cholesterol concentration is expressed as mg./100 ml. of blood serum and liver bile. Results are means ±S.D. for 20 rats in each group.

Acid	Control group	Inanition group	Pyridoxine-deficient group
Iodine value of Bloor extract	42·0 ±1·7	39·5 ±2·1	21·0 ±6·6
Oleic acid (%)	31·65±1·3	27·10±1·8	36·1 ±3·6
Dienoic acid (%)	41·33±3·5	38·10±3·2	48·33±2·7
Trienoic acid (%)	1·38±0·4	0·98±0·2	1·16±0·4
Tetraenoic acid (%)	5·71±0·8	4·42±0·5	2·26±0·9
Pentaenoic acid (%)	0·97±0·3	0·78±0·2	0·69±0·3
Hexaenoic acid (%)	0·53±0·2	Traces	Traces
Serum cholesterol, free	43·0 ±4·8	38·2 ±2·1	80·0 ±3·1
Serum cholesterol, ester	62·0 ±2·7	55·0 ±4·1	118·0 ±5·3
Bile cholesterol	12·4 ±0·2	9·1 ±0·3	10·6 ±1·4

concentration. Our data, however, do not show any significant difference of the unsaturated fatty acid spectrum of aorta between pyridoxine-deficient rats and the controls.

The hypercholesterolaemia of pyridoxine deficiency is not a reflexion of any aberration in the synthetic pattern of cholesterol by liver, as evidenced by the constancy of bile cholesterol. If the enzymic pattern for cholesterol synthesis is not affected by pyridoxine deficiency, it may tentatively be assumed that cholesterol synthesis by arteries is also normal and the cholesterol that is deposited in the intima has reached there from the blood stream.

Although the polyethenoid fatty acid, particularly arachidonic acid content, of the serum was lowered in pyridoxine deficiency, the rats did not show symptoms of deficiency of essential fatty acids to any significant extent, as judged by their general appearance. The same order of tetraenoic fatty acid concentration in the aortae of the control and of the deficient rats, despite the significant difference in the blood concentrations of the two groups of animals, points either to a relatively unimportant role of arachidonic acid in the aortae or to a greater effort of the deficient animals to maintain the tetraenoic acid content of the aortae constant. In either case it is difficult to reconcile it with the hypothesis of Sinclair (1956).

SUMMARY

1. Pyridoxine deficiency induces a significant degree of hypercholesterolaemia in rats and there is deposition of cholesterol in aortae (thoracic and abdominal).

2. The tetraenoic fatty acid level of serum is lowered in pyridoxine-deficient hypercholesterolaemia in rats, but the unsaturated fatty acid spectrum of aortae is not significantly altered in pyridoxine deficiency.

3. The hypercholesterolaemia is probably due to increased mobilization of cholesterol from the liver.

The authors are grateful to C.S.I.R. for a grant-in-aid and to Principal K. C. Mukherjee for interest and advice.

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The Inhibition of Photoreactions of Chloroplasts by 2-Alkyl-4-hydroxyquinoline *N*-Oxides

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(*Received* 4 *July* 1960)

An antagonist of streptomycin action in several bacteria was shown to consist of a mixture of 2-alkyl-4-hydroxyquinoline *N*-oxides (Cornforth & James, 1956). The same compounds were demonstrated to be effective inhibitors of electron transport in cell-free preparations of heart muscle and some bacterial preparations (Lightbown & Jackson, 1956; Jackson & Lightbown, 1958). The site of the inhibitory action of 2-heptyl-4-hydroxyquinoline *N*-oxide in heart-muscle preparations was concluded to be between diaphorase and cytochrome *c*, and most probably between cytochrome *b* and cytochrome *c* (Jackson & Lightbown, 1958).

More recently, the same compound was observed (Smith & Baltscheffsky, 1959) to inhibit completely at 1 μM concentration light-induced phosphorylation in extracts of the photosynthetic bacterium *Rhodospirillum rubrum*. At the same concentration it had no effect on the respiratory activity of the extract. In addition, it produced changes in the light-dependent difference spectrum which were interpreted as an increased oxidation of cytochrome c_2 and a decreased oxidation of the *b*-type cytochromes. 2-Heptyl-4-hydroxyquinoline *N*-oxide has also been used as an accelerator of photooxidation (Vernon, 1959) and inhibitor of photoreduction (Vernon & Ash, 1959) catalysed by bacterial chromatophores. A communication describing its inhibition of photophosphorylation catalysed by spinach chloroplasts has recently appeared (Baltscheffsky, 1959).

In view of the potency of these compounds in specifically inhibiting the bacterial photophosphorylative system, it was thought of importance to determine their effect on the different photoreactions catalysed by higher-plant chloroplasts. Two of the most potent of this group of inhibitors were selected (Lightbown & Jackson, 1956), and a quantitative study of their inhibitory action was undertaken. Experiments designed to test their specificity, site of action, manner of attachment and relation to other inhibitors of photoreactions in chloroplasts are described below.

METHODS

Preparation of chloroplasts. Chloroplasts and chloroplast fragments were prepared from greenhouse-grown Swiss-chard leaves as described by Avron (1960).

Measurement of reactions. The procedure followed and the measurement of the production of adenosine [^{32}P]triphosphate (AT^{32}P) were as described by Avron (1960). The reduction of ferricyanide was determined from the decrease of E at 420 mμ of a sample of the trichloroacetic acid-denatured supernatant, a molar absorption coefficient of $1{\cdot}06 \times 10^3$ being used. The reduction of triphosphopyridine nucleotide (TPN) and trichloroindophenol was determined from the change of E at 340 and 620 mμ respectively of the complete reaction mixture. All changes in E were measured against a blank which contained the same reaction mixture, but to which no dye had been added.

All reactions were run at 17°, with air as the gas phase. The reaction cuvettes were placed within the aquarium described by Avron (1960), with an illumination of about 150,000 lux of white light.

Solutions of inhibitor. The inhibitor was dissolved in mM-NaOH solution and added as such. Control samples contained an equivalent amount of mM-NaOH. The concentration of the inhibitor in the stock solution was determined before each experiment by its absorption at 346 mμ (Corn-

forth & James, 1956). The latter procedure was found to be essential since the absorption decreased considerably during a few days in storage. Usually a fresh solution was prepared for each experiment.

RESULTS

The effects of several concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide on the reduction of ferricyanide and the associated phosphorylation (Avron, Krogmann & Jagendorf, 1958) are illustrated in Fig. 1. Both the phosphorylation and the reduction were inhibited to a similar extent. In general, the phosphorylation seemed to be slightly more sensitive to these inhibitors than the reduction. This was true with either ferricyanide or TPN as the electron acceptor.

Fig. 1 also demonstrates the fast action of these inhibitors. There was never any increase in inhibition observed as the reaction progressed, indicating that the interaction of the inhibitors with the active site in the chloroplasts was rapid and did not require a preincubation period. This is particularly noteworthy in view of the extremely low concentrations of these compounds required for inhibition.

The effect of increasing concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide was examined on the following types of photoreactions catalysed by higher-plant chloroplasts: (1) photophosphorylation dependent upon the presence of one of several cofactors (Jagendorf & Avron, 1958); (2) reduction of trichloroindophenol in the Hill reaction; (3) reduction of TPN or ferricyanide, and accompanying phosphorylations (Avron & Jagendorf, 1959). It is clear that these reactions fall into three groups (Fig. 2). The phenazine methosulphate-dependent photophosphorylation comprises the first, least-sensitive group. Concentrations up to 20 μM have little or no inhibitory effect in this reaction. Intermediate sensitivity was shown by most of the reactions tested, with 50% inhibition occurring around 10 μM. In this intermediate range fall the photophosphorylations dependent upon the presence of flavin mononucleotide and vitamin K_3 (Fig. 2). In addition, the photophosphorylation

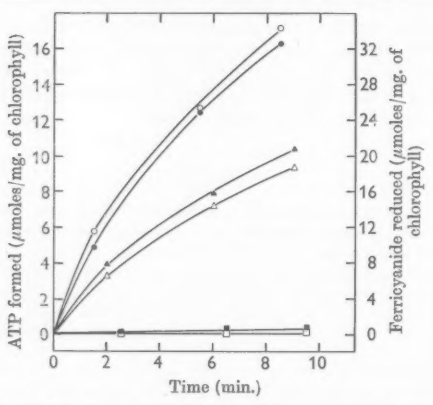

Fig. 1. Time course of inhibition of ferricyanide reduction and accompanying phosphorylation. Reaction mixture contained (μmoles): 2-amino-2-hydroxymethylpropane-1:3-diol–HCl buffer (pH 7·8), 45; NaCl, 60; $MgCl_2$, 12; sodium and potassium phosphate (pH 7·8), 12 (containing 4×10^5 counts/min. of ^{32}P); ADP (pH 7·8), 8; $K_3Fe(CN)_6$, 4; 2-heptyl-4-hydroxyquinoline *N*-oxide, as indicated below; and chloroplasts washed once, in a total volume of 3·0 ml. Chloroplasts contained 29 μg. of chlorophyll/ml. of reaction mixture. Open symbols represent the production of ATP (left ordinate). Closed symbols represent the reduction of ferricyanide (right ordinate). ○, ●, No inhibitor added; △, ▲, 0·6 μM-2-heptyl-4-hydroxyquinoline *N*-oxide; □, ■, 6 μM-2-heptyl-4-hydroxyquinoline *N*-oxide.

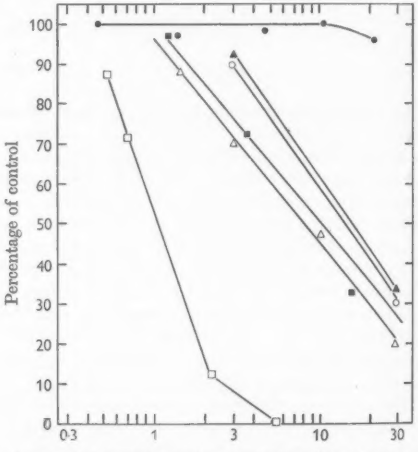

Fig. 2. Effect of increasing concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide on chloroplast photoreactions. Reaction mixture was as described in Fig. 1, except for the substitution of the compounds indicated below for ferricyanide. Chloroplasts contained 14 μg. of chlorophyll/ml. Reaction time: 5 min., except for trichloroindophenol reduction, which was measured over a period of 15 sec. ●, 33 μM-Phenazine methosulphate; ▲, 33 μM-menadione (vitamin K_3); ○, 33 μM-flavin mononucleotide; ■, 0·167 mM-TPN; △, 33 μM-trichloroindophenol; □, 0·67 mM-$K_3Fe(CN)_6$. In the reaction containing trichloroindophenol, the reduction of the dye is represented; in all others the production of ATP is marked. Control activity on the ordinate corresponds to the following values (μmoles of ATP formed/mg. of chlorophyll/hr.): phenazine methosulphate, 1840; menadione, 146; flavin mononucleotide, 153; TPN, 24; $K_3Fe(CN)_6$, 205. The control activity of trichloroindophenol reduction was 286 μmoles reduced/mg. of chlorophyll/hr.

dependent upon indigo carmine, reduced trichloroindophenol (Krogmann & Vennesland, 1959; with our preparations this reaction did not show a strict oxygen-dependence) and the low phosphorylation present in the absence of any cofactor (Avron, 1960), fall in this intermediate category (not shown in the Figure). The Hill reaction, with trichloroindophenol as an electron acceptor, and the reduction and accompanying phosphorylation of TPN, could also be placed with the intermediate group.

The reaction most sensitive to 2-heptyl-4-hydroxyquinoline *N*-oxide was the reduction, and accompanying phosphorylation, of ferricyanide. Here, 50% inhibition was reached at a concentration of about 1 μM. As shown in Fig. 1, the inhibition of the reduction of ferricyanide and the accompanying phosphorylation were similar.

Fig. 3 illustrates the data obtained when the heptyl derivative was replaced by its more potent analogue 2-nonyl-4-hydroxyquinoline *N*-oxide. In all these reactions, as in its action in antagonizing dihydrostreptomycin activity (Lightbown & Jackson, 1956), the nonyl derivative was about five to ten times as effective as the heptyl compound. Here again, phenazine methosulphate-dependent phosphorylation was least sensitive, but small inhibitions could be observed above a concentration of 5 μM. The same reactions fell into the intermediate-sensitivity group, as with the heptyl derivative, but 50% inhibition was obtained at about 1 μM concentration. Also the photophosphorylation in the absence of any cofactor, and those dependent upon the presence of indigo carmine and reduced trichloroindophenol, were intermediate in sensitivity. The reduction of ferricyanide and the accompanying phosphorylation were most sensitive; 50% inhibition was reached at about 0·2 μM-2-nonyl-4-hydroxyquinoline *N*-oxide.

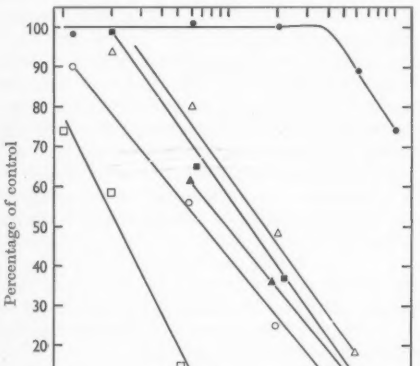

Fig. 3. Effect of increasing concentrations of 2-nonyl-4-hydroxyquinoline *N*-oxide on chloroplast photoreactions. Details and symbols are as described in Fig. 2. Chloroplasts contained 19 μg. of chlorophyll/ml. Control activity on the ordinate corresponds to the following values (μmoles of ATP formed/mg. of chlorophyll/hr.): phenazine methosulphate, 1620; menadione, 161; flavin mononucleotide, 222; TPN, 18; $K_3Fe(CN)_6$, 191. The control activity of trichloroindophenol reduction was 276 μmoles reduced/mg. of chlorophyll/hr.

Table 1. *Dependence of inhibition of phosphorylation by 2-heptyl-4-hydroxyquinoline N-oxide upon chloroplast concentration*

Reaction mixture was as described under Fig. 1. Reaction time, 5 min. Control values: (*a*) phosphorylation in μmoles of ATP formed/mg. of chlorophyll/hr., 107, 131 and 120 with 6·9, 13·9 and 26·8 μg. of chlorophyll respectively; (*b*) reduction in μmoles of ferricyanide reduced/mg. of chlorophyll/hr., 232, 290 and 230 with 6·9, 13·9 and 26·8 μg. of chlorophyll respectively.

Chloroplast concn. (μg. of chlorophyll/ml.)	2-Heptyl-4-hydroxyquinoline *N*-oxide concn. (μM)	Percentage of control	
		Phosphorylation	Reduction
6·9	0·4	53	61
13·9	0·4	80	75
26·8	0·4	91	100
26·8	0·8	76	87

Variation in some of the early experiments on the action of these inhibitors was traced to the concentration of the chloroplasts in the experimental flasks (Table 1). Doubling the concentration of the chloroplasts lowered the inhibition of the photophosphorylation dependent upon ferricyanide reduction from 47% to 20%. Further doubling of the chloroplasts concentration decreased it to 9%. It was therefore important to compare the extent of inhibitions of different reactions at similar chloroplast concentrations, whenever possible.

The effects of the heptyl- or nonyl-hydroxyquinoline *N*-oxides were also tested with chloroplast fragments (Avron, 1960) as well as whole chloroplasts. The reactions studied were the photophosphorylations dependent upon phenazine methosulphate, flavin mononucleotide, indigo carmine, reduced indophenol and no cofactor, and the reduction and accompanying phosphorylation of ferricyanide. In no case was there any substantial difference between these two types of preparations.

Since ferricyanide-dependent photoreactions were most severely inhibited, it was thought desirable to eliminate the possibility of a direct interaction between ferricyanide and the inhibitors as a factor in their increased susceptibility. The effect of

2-heptyl-4-hydroxyquinoline *N*-oxide was tested therefore on the action of reduced triphosphopyridine nucleotide (TPNH) diaphorase (Avron & Jagendorf, 1956), isolated from the same leaves, with ferricyanide or trichloroindophenol acting as electron acceptors. No inhibition by 15 μM-2-heptyl-4-hydroxyquinoline *N*-oxide of TPNH diaphorase was observed with either electron acceptor.

The sensitivity of the different photoreactions to 2-heptyl-4-hydroxyquinoline *N*-oxide and 2-nonyl-4-hydroxyquinoline *N*-oxide was similar to that previously observed with another potent inhibitor, 3-(*p*-chlorophenyl)-1:1-dimethylurea (Jagendorf & Avron, 1959). This similarity is evident in the resistance exhibited by the phenazine methosulphate-mediated phosphorylation to both inhibitors, as compared with the other photoreactions studied. Since the site of action of 3-(*p*-chlorophenyl)-1:1-dimethylurea has been indicated by several different types of experiments to be on the oxygen-evolution path (Bishop, 1958; Jagendorf & Avron, 1959; Vernon & Zaugg, 1960), several experiments were designed to test further the identity in site of action of the two inhibitors.

It was previously shown (Avron & Jagendorf, 1959) that although phenazine methosulphate-dependent phosphorylation was insensitive to 3-(*p*-chlorophenyl)-1:1-dimethylurea when added alone, it was completely inhibited by the same concentration of 3-(*p*-chlorophenyl)-1:1-dimethylurea when added with small amounts of ferricyanide. In this respect, too, 2-heptyl-4-hydroxyquinoline *N*-oxide behaved similarly to 3-(*p*-chlorophenyl)-1:1-dimethylurea; the reaction was completely inhibited in the presence of ferricyanide and 2-heptyl-4-hydroxyquinoline *N*-oxide, but not in the presence of either compound alone (Fig. 4). If 3-(*p*-chlorophenyl)-1:1-dimethylurea and the quinoline *N*-oxides act in the identical site on the chloroplasts, it would be expected that their action would be additive. That is, when a concentration of 3-(*p*-chlorophenyl)-1:1-dimethylurea was added to one of 2-nonyl-4-hydroxyquinoline *N*-oxide, each of which caused the same inhibitory effect when added alone, it would be expected that the resulting inhibition would be equivalent to that produced by doubling the concentration of each inhibitor by itself. If, on the other hand, the two were acting at two different sites on the same chain, the decrease in activity caused by adding the two inhibitors together should not exceed that which was produced by each one by itself. Table 2 shows the results of the experiment described. The inhibition produced by the presence of both inhibitors together was equivalent to that produced by double the amount of each by itself, pointing to the identity of their site of attack.

The reversal, by washing, of the inhibitory effect of 3-(*p*-chlorophenyl)-1:1-dimethylurea has been previously described (Wessels & Van Der Veen, 1956; Spikes, 1956). The effect of the quinoline *N*-oxides was, similarly, easily reversed by washing of the chloroplast preparation; 80–90 % of the activity was recovered after a single wash of chloroplasts treated with 0·5 μM-2-nonyl-4-hydroxyquinoline *N*-oxide.

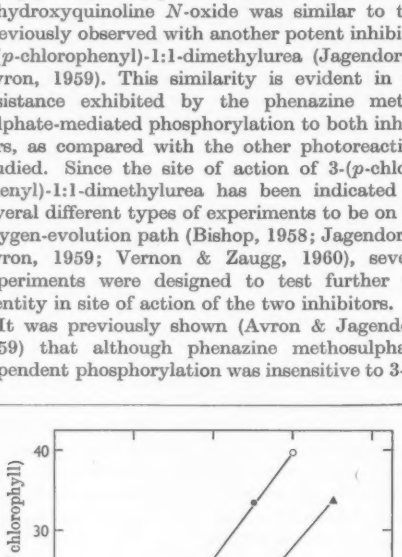

Fig. 4. Effect of ferricyanide on the inhibition of phenazine methosulphate-dependent photophosphorylation by 2-heptyl-4-hydroxyquinoline *N*-oxide. Reaction mixture was as described under Fig. 1, except for the substitution of 33 μM-phenazine methosulphate for $K_3Fe(CN)_6$. Chloroplasts contained 48 μg. of chlorophyll/ml. of reaction mixture. ●, Control; ○, 3·2 μM-2-heptyl-4-hydroxyquinoline *N*-oxide; ▲, 0·33 mM-$K_3Fe(CN)_6$; △, 3·2 μM-2-heptyl-4-hydroxyquinoline *N*-oxide and 0·33 mM-$K_3Fe(CN)_6$.

Table 2. *Additive inhibitory action of 2-nonyl-4-hydroxyquinoline N-oxide and 3-(p-chlorophenyl)-1:1-dimethylurea on photophosphorylation*

Reaction mixture was as described under Fig. 1. Reaction time, 5 min. Control value: 126 μmoles of ATP/mg. of chlorophyll/hr. Once-washed chloroplasts, containing 16 μg. of chlorophyll/ml., were used.

Additions (μM)		
2-Nonyl-4-hydroxyquinoline *N*-oxide	3-(*p*-Chlorophenyl)-1:1-dimethylurea	Percentage of control
0	0	100
0·3	0	25
0	1·0	22
0·3	1·0	5
0·6	0	3
0	2·0	7

DISCUSSION

The inhibitory effect of the 2-alkyl-4-hydroxyquinoline *N*-oxides in the photoreactions catalysed by chloroplasts was quite specific. The inhibitors could not directly interact with the phosphorylation mechanism or the electron-transport steps closely associated with it. This was concluded since the most rapid photophosphorylation reaction known (i.e. in the presence of phenazine methosulphate) was by far the least sensitive. Their site of action must be directly involved therefore in the electron-transport steps necessary for the Hill reaction and the photophosphorylations requiring cofactors other than phenazine methosulphate, but only indirectly, if at all, in the photophosphorylation catalysed by phenazine methosulphate.

The similarity between the pattern of inhibitory action of 3-(*p*-chlorophenyl)-1:1-dimethylurea (Jagendorf & Avron, 1959) and the quinoline *N*-oxides (Figs. 2, 3), and the additivity of their action (Table 2), suggest that the site of action of the two is identical. It would seem therefore that the heptyl- and nonyl-hydroxyquinoline *N*-oxides inhibit a step in the path of oxygen evolution.

The reduction and accompanying phosphorylation of ferricyanide were most severely affected by the inhibitors studied. In this case, 2-nonyl-4-hydroxyquinoline *N*-oxide, which reduces the control rate by 50 % at 0·2 μM, would be equal in its potency to the most active inhibitors of the reaction known (Wessels & Van der Veen, 1956). It may be worth emphasizing that at 0·2 μM the concentration of the inhibitor was several-fold lower than that of chlorophyll in the reaction mixture, even though the concentration of the chloroplasts used was very low and limiting the rate of the reaction. This supports the suggestion that only part of the chlorophyll within the chloroplast is active in catalysing the photoreaction studied.

The reason for the high sensitivity of the ferricyanide-dependent reactions, as compared with the others investigated, is not clear. However, the manner in which Figs. 2 and 3 were drawn tends to obliterate the fact that these reactions differ considerably in their rates. In terms of μmoles of ATP synthesized/mg. of chlorophyll/hr. the photophosphorylation dependent on phenazine methosulphate has a rate of 2000, on ferricyanide 200, and on TPN 20. In this light, the resistance of the photophosphorylation dependent on phenazine methosulphate becomes even more significant, and the difference between those dependent on ferricyanide and TPN may only be a reflexion of the difference in the rates of the respective reactions.

SUMMARY

1. 2-Heptyl-4-hydroxyquinoline *N*-oxide and 2-nonyl-4-hydroxyquinoline *N*-oxide were found to be potent inhibitors of some of the photoreactions catalysed by Swiss-chard chloroplasts. Their quantitative effects are reported on the Hill reaction, cyclic photophosphorylation in the presence of different catalysts and open photophosphorylation in the presence of ferricyanide or triphosphopyridine nucleotide.

2. By far the most resistant reaction was the cyclic photophosphorylation dependent upon the presence of phenazine methosulphate. The most sensitive were the reduction and associated phosphorylation of ferricyanide. Inhibition of the latter by 50 % was observed with 0·2 μM concentrations of the nonyl derivative.

3. By analogy with the inhibition caused by 3-(*p*-chlorophenyl)-1:1-dimethylurea, it is suggested that the quinoline *N*-oxides react with a site on the oxygen-evolution pathway.

I would like to thank Dr M. Kogut and Dr J. W. Lightbown of the National Institute for Medical Research, Mill Hill, London, for generously supplying the samples of the quinoline *N*-oxides used in this work.

The excellent technical assistance of Mrs F. Itzhak is gratefully acknowledged.

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Fatty Acids in Semen

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(*Received* 23 *May* 1960)

Only one paper seems to have appeared on the presence of free fatty acids in semen, namely that of Flipse & Potter (1955), who identified acetic acid and formic acid in bull semen. We now report the results of investigations on the concentrations of volatile fatty acids and free fatty acids (long-chain) in the semen of bull, ram, rabbit, fowl, dog, stallion and man. In addition, volatile fatty acid concentrations in the semen and blood of rams have been compared, and their concentration determined in ram-epididymal fluid.

EXPERIMENTAL

Materials. Ram semen was collected by electrical stimulation by the bipolar electrode of Blackshaw (1954). An artificial vagina was used for collecting bull, stallion and rabbit semen, with White's (1955) apparatus for the last named. Dog semen was obtained by manipulation and fowl semen by abdominal massage (Burrows & Quinn, 1939). In each experiment, the semen was frozen in solid CO_2 and ejaculates were usually pooled for analysis. Normal human semen was obtained from a sterility clinic.

Testes were dissected from rams immediately on slaughter and packed in an ice–salt mixture during transport to the laboratory. Testicular fluid was aspirated from the rete testes and fluid taken from the tail of the epididymis after cutting with a scalpel. Pooled material from 10 rams was used in the analysis.

Blood samples were obtained from the jugular vein, with heparin as anticoagulant.

Determination of volatile fatty acids. Volatile fatty acids were estimated by the method described by Annison (1954). Blood or semen (1 vol.) was mixed with 3·5 vol. of water and deproteinized with 0·5 vol. of freshly prepared 25% (w/v) metaphosphoric acid. The precipitate was separated by centrifuging and washed with 2·5 vol. of 2·5% (w/v) metaphosphoric acid. The supernatant and washings were adjusted to pH 8·0–8·5, with 10N-NaOH, by using phenolphthalein as indicator, and concentrated where necessary *in vacuo* to 3–4 ml. at 40° in a rotary evaporator. A few drops of octan-2-ol were added to prevent the frothing of semen samples, which became excessive if the temperature rose above 40°. The concentrated solution was adjusted to pH 2·5–3·0 with 10N-H_2SO_4 (thymol blue indicator) and transferred to a Markham still for distillation. Two successive 100 ml. distillates were collected (the second served as a blank) and titrated with 0·01N-NaOH (phenolphthalein indicator) under CO_2-free conditions.

The volatile fatty acids in the neutralized distillates from the semen samples were analysed by gas–liquid chromatography (James & Martin, 1952) according to the general procedure outlined by Annison (1954).

Determination of free fatty acids. Semen was diluted with 2 vol. of water, and portions (2 ml.) were examined for free fatty acid (long-chain) by the method of Dole (1956), with modifications described by Annison (1960). Dilution of semen before extraction with organic solvents was necessary to avoid the formation of stable emulsions.

RESULTS

Values for the volatile fatty acid content of the semen of the bull, ram, rabbit, man, stallion, fowl and dog are given in Table 1. Since many of the

Table 1. *Volatile fatty acid content of the semen of various species*

Analyses for individual acids were usually made on single pooled samples.

Animal	No. of samples	Total (m-equiv./l.)	Individual acids (molecular percentages)	
			Formic acid	Acetic acid
Bull	17	0·82 (0·20–1·43)	27	73
Ram	22	1·63 (0·68–2·73)	0	100
Rabbit	2	1·34 (1·08–1·80)	4	96
Man	3	0·73 (0·50–0·95)	0	100
Stallion	4	0·77 (0·67–1·00)	0	100
Fowl	2	0·51 (0·37–0·64)	0	100
Dog				
(1) Prostatic fluid	4	0·11 (0·06–0·15)	—	—
(2) Spermatozoa fraction	2	0·10 (0·08–1·20)	0	100
(3) Pre-spermatozoa fraction	1	0·90	—	—

samples were pooled ejaculates, the normal range of values for individuals in any one species is probably greater than the data indicate. However, values as high as those recorded by Flipse & Potter (1955) in bull semen (i.e. up to 25·0 m-equiv./l.) were never encountered.

Table 2 shows a comparison of the volatile fatty acid concentration in the blood and semen of 20 rams. Blood samples were taken at the same time as the semen from each animal and four animals from each of five strains were used.

Analyses of variance for the blood and semen data did not reveal any statistically significant differences in volatile fatty acid concentration between strains. In all animals the concentration of these acids in semen was higher than in the blood (t 18·2, $P < 0·01$). There was, however, no significant correlation between the two (r 0·112; D.F. 18).

The volatile fatty acid content of testicular and tail fluids, pooled in each instance from 10 ram epididymes, was 1·93 and 1·70 m-equiv./l. respectively.

The free fatty acid (long-chain) content of the semen of the bull, ram, stallion, rabbit and dog is shown in Table 3. Plasma-free fatty acid analyses were not undertaken, but reported values for the dog, sheep and rabbit (cf. Annison, 1960) suggest that the semen values were lower than those of plasma. One sample of dog prostatic fluid contained considerably less than did the spermatozoa fraction from the same ejaculate (Table 3).

DISCUSSION

The results reported here for bull semen are difficult to reconcile with those of Flipse & Potter (1955), who found volatile fatty acid concentrations in bull semen of 7·9–25·0 m-equiv./l., i.e. approximately ten times those found in the present investigation. The discrepancy cannot be explained in terms of the age of the semen samples before analysis since Flipse & Potter (1955) found no consistent differences in total or constituent acids when semen samples were analysed 5 and 180 min. after collection. Further, many of the samples analysed in the present study were cooled immediately with solid carbon dioxide, so that, in these instances, there could be no question of a breakdown of the volatile fatty acids before analysis. The relative concentrations of acetic acid and formic

Table 2. *Comparison of the volatile fatty acid concentration (m-equiv./l.) in the blood and semen of 20 merino rams (four animals of each strain)*

Mean values are given, with the range for each strain and the overall population S.D. The residual mean square is shown in italics.

Strain	Blood	Semen
Peppin A	0·84 (0·63–1·27)	1·71 (1·04–2·36)
Peppin B	0·91 (0·72–1·03)	1·66 (1·03–2·25)
Non-peppin medium	0·67 (0·59–0·76)	1·06 (0·68–1·48)
Fine wool	0·68 (0·62–0·77)	1·40 (1·10–1·75)*
S.A. strong	0·60 (0·52–0·68)	1·67 (1·44–2·23)
Overall	0·74±0·17	1·50±0·46

Analysis of variance

	Blood		Semen	
Source of variation	D.F.	Variance ratio	D.F.	Variance ratio
Between strains	4	2·5	4	2·7
Residual	15	*0·264*	14†	*0·118*

* Three animals only.

† Decreased by 1 to compensate for the insertion of a missing value, by the method of Cochran & Cox (1950).

Table 3. *Free fatty acids in secretions of several species.*

Each sample usually consisted of several ejaculates.

Animal	Secretion	No. of samples	Concentration (m-equiv./l.)
Bull	Semen	3	0·13 (0·06–0·24)
Stallion	Semen	2	0·10 (0·10–0·09)
Ram	Semen	6	0·21 (0·15–0·27)
Rabbit	Semen	1	0·01
Dog	Spermatozoa fraction	1	0·18
Dog	Prostatic fluid	1	0·02
Fowl	Seminal plasma	1	0·08

acid in bull semen in the present studies were similar to those reported by Flipse & Potter (1955).

There is evidence that bull spermatozoa are able to oxidize acetic acid (Lardy & Phillips, 1944), and if this was present in seminal plasma in the relatively high concentrations reported by Flipse & Potter (1955) it could clearly be an important source of energy for the spermatozoa after ejaculation. The concentrations found here would, however, be of little metabolic significance in comparison with the large quantities of fructose that are known to be present normally in bull semen (Mann, 1946, 1954).

Much of the carbohydrate ingested by herbivores is converted into volatile fatty acids by micro-organisms in the alimentary tract, and, in ruminants at least, volatile fatty acid, rather than sugar, is the chief form in which carbohydrate is absorbed (Annison & Lewis, 1959). It was thought therefore that acetate concentrations in the semen of herbivores (particularly the ram and bull) might be higher than those of other species such as man, dog and fowl. There are, however, no striking differences between species.

The fact that the concentration of volatile fatty acids is consistently higher in semen than in blood suggests that the accessory organs may be capable of concentrating or producing the acids.

The concentrations of volatile fatty acids in the epididymal fluids examined were of the same order as that of whole semen. However, the volatile fatty acids of the epididymal tail may well be in a dynamic state, with a constant interchange between blood, epididymal plasma and spermatozoa. These substances might still therefore be important in the economy of the spermatozoa despite their low concentration in the epididymal fluid.

An unexpected finding was the absence of formate from the semen of all species examined except bull and rabbit. Formate is present in the blood of sheep, goats, oxen, rabbits, horses, dogs and in the human blood (Annison, 1954).

Free fatty acids have been shown to be the metabolically active fraction of circulating lipids (cf. Frederickson & Gordon, 1958). Although they are present in low concentrations in blood (0·3–0·8 m-equiv./l.), rapid turnover rate is an indication of their metabolic importance. The concentrations found in semen (Table 3) are such that free fatty acids can hardly be important as an energy source to spermatozoa after ejaculation. It is possible, however, that they are involved in the metabolism of spermatozoa in the epididymis.

SUMMARY

1. The concentrations of volatile fatty acids and free fatty acids (long-chain) in bull, ram, rabbit, fowl, dog, stallion and human semen were determined. The volatile fatty acid concentration ranged from 0·08 to 2·73 m-equiv./l. and there were no outstanding differences between ruminants and non-ruminants. Values for free fatty acid content ranged from 0·01 m-equiv./l. for dog prostatic fluid to 0·21 m-equiv./l. for ram semen.

2. Chromatographic evidence indicated that acetic acid and formic acid were present in bull and rabbit semen, but in the other species examined acetic acid was the only volatile fatty acid detected.

3. No significant correlation was found between the volatile fatty acid concentrations in the semen and blood of rams.

4. The volatile fatty acid concentration of ram epididymal fluid was similar to that of whole semen.

5. It is concluded that volatile fatty acid and free fatty acid (long-chain) concentrations in the semen of the species examined is too low to be of importance for the spermatozoa as a substrate after ejaculation.

We are indebted to Professor C. W. Emmens for his interest and advice, to Mr P. Bryden of the N.S.W. State Abbatoirs for the supply of ram testes, and to the Wool Research Committee for financial assistance. The work of two authors (I.G.W. and T.W.S.) has also been aided by grants from the Planned Parenthood Federation of America and the Rural Credits Development Fund of the Commonwealth Bank of Australia.

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Protective Influence of Hydrolysed Product of 'Glucose Cycloacetoacetate' in Experimental Anaemia Resulting from Necrogenic Diet

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(*Received* 1 *June* 1960)

Nath & Prasannan (1959) have shown that hydrolysed 'glucose cycloacetoacetate' can restore the erythrocyte count and haemoglobin level with the same efficacy as vitamin B_{12} in experimental anaemia induced by phenylhydrazine. The occurrence of anaemia of some type or other due to the liver damage is well known (Shumacker & Wintrobe 1938; Koszewski, 1952).

During the development of dietary liver necrosis it is recognized that depletion of reduced glutathione in liver occurs (Leaf & Neuberger, 1947; Lindon & Work, 1953).

Nath & Behki (1958) have shown that the depletion of glutathione occurring in experimental diabetes induced by acetoacetate in rabbits can be checked by administering the sodium salt of glucose cycloacetoacetate. The same substance also checked the depletion of glutathione in scorbutic guinea pigs (Behki, Motlag & Nath, 1958).

The effect of glucose cycloacetoacetate, its hydrolysed product and vitamin B_{12} on haemoglobin level and erythrocyte count has been studied during the feeding of necrogenic diet to rats by the methods of Lindon & Work (1953), with slight modifications.

EXPERIMENTAL

Animals and diets. Weanling male albino rats weighing 40-60 g. were divided into the following five groups in respect of their diet and experimental treatment. Each group contained six or seven animals. The grouping of the animals was carried out in such a manner that the average body weight of the animals for all groups was approximately the same. Group I animals were fed normal stock diet (%): casein 12, cane sugar 36, groundnut oil 6·4, arrowroot 40, cod-liver oil 1·6, salt mixture 3·5 (Hawk, Oser & Summerson, 1947), L-cystine 0·3 and choline chloride 0·2. The following vitamins were added/kg. of the above diet: thiamine hydrochloride 5 mg., riboflavin 5 mg., pyridoxine 5 mg., *p*-aminobenzoic acid 5 mg., niacin 5 mg., calcium-D-pantothenate 25 mg., inositol 50 mg., biotin 0·1 mg., folic acid 1 mg., vitamin B_{12} 25 μg., α-tocopherol acetate 25 mg. and vitamin K 5 mg. All the animals in the remaining groups were fed on the necrogenic diet (Lindon & Work, 1953) with minor modification as follows (%): baker's yeast 6, cane sugar 36, arrowroot 46, cod-liver oil 1·6, ground-nut oil 6·4, salt mixture 3·5 (Hawk *et al.* 1947). All the vitamins except α-tocopherol and vitamin B_{12} were added as mentioned for the diet of group I animals. Group III animals were given together with their diet crystalline glucose cycloacetoacetate at a concn. of 0·3%. Group IV animals were injected subcutaneously with 1 g.equiv. of glucose cycloacetoacetate (hydrolysed)/kg. body wt. on alternate days. Group V animals were injected subcutaneously, on alternate days, with vitamin B_{12} (BE-Douze Lab. Grimault, Paris, 200 μg./kg. body wt).

Analytical methods. Blood was taken from the tail before the animal was fed. The tip of the tail was shaved clean, washed with saturated solution of sodium citrate and dried with sterile cotton. A small longitudinal incision (1 mm.) was made on one of the tail veins, and the first drop of blood was wiped off with moistened cotton. By applying gentle pressure with fingers, the freely flowing blood was collected into the clean pipettes. The erythrocyte count was made by the usual procedure with a Neubauer Ruling (improved double Neubauer Ruling Depth 0·100 mm., 0·01 sq.mm.) Haemoglobin was estimated by the method of Wong (1928) with 0·2–0·5 ml. of blood by converting it into Fe^{3+} ions with conc. H_2SO_4 and potassium persulphate and by using 3N-KSCN for colour development. The extinction of the coloured sample was compared with that of the standard iron solution with a green filter in a photoelectric colorimeter (Unicam, Cambridge, England). The haemoglobin was calculated from the corresponding blood iron value taking 0·34% iron content for haemoglobin.

Glucose cycloacetoacetate was first prepared according to a method of West (1927) modified by Nath, Chitale & Belavady (1952), which was then hydrolysed as follows: In a 50 ml. conical flask, 2 g. of crystalline glucose cycloacetoacetate was hydrolysed with 10 ml. of 2N-HCl over a boiling-water bath for 20 min. The flask was cooled over ice, and the contents were then neutralized to about pH 7. It was then extracted three times with ether; the lower aqueous fraction was separated and adjusted carefully to pH 7·2 with a few drops of 0·1N-NaOH, by using Merck special indicator paper (pH range 6·6–8·0).

RESULTS AND DISCUSSION

In the course of 35 days, the animals fed on normal stock diet showed a slight increase in erythrocyte count and haemoglobin, whereas those fed on necrogenic diet showed a considerable decrease, the erythrocyte count and haemoglobin being decreased to levels corresponding to 64 and 66% of the initial average values respectively (see Table 1). The presence of glucose cyclo-

Table 1. *Antianaemic effect of hydrolysed glucose cycloacetoacetate and vitamin B_{12} in rats receiving the necrogenic diet*

RBC, red blood corpuscles; Hb, haemoglobin.

Group no.	Mean values from seven rats of RBC (10^6/mm^3.) and Hb (g./100 ml.) and standard deviation after time: 0	1 week	3 weeks	5 weeks
I. Normal stock diet				
RBC	7·68±1·12	8·30±0·65	8·74±0·66	8·89±0·69
Hb	12·5 ±1·21	13·1 ±0·89	13·5 ±0·94	14·3 ±1·25
II. Necrogenic diet				
RBC	8·57±1·13	8·37±1·08	7·84±0·85	5·55±0·57
Hb	14·4 ±1·15	13·8 ±0·82	12·4 ±1·21	9·6 ±1·03
III. Necrogenic diet + glucose cycloacetoacetate at a concn. of 0·3% in the diet				
RBC	7·44±0·83	7·13±1·27	6·28±0·83	5·39±0·75
Hb	13·5 ±1·85	13·2 ±1·55	11·4 ±1·19	10·2 ±1·16
IV. Necrogenic diet + hydrolysed glucose cycloacetoacetate injected on alternate days (100 mg./100 g. body wt.)				
RBC	7·93±1·03	8·32±0·75	8·02±0·94	8·00±0·35
Hb	13·4 ±1·16	13·5 ±1·02	13·8 ±1·93	13·5 ±1·78
V. Necrogenic diet + vitamin B_{12} injected on alternate days (20 μg./100 g. body wt.)				
RBC	8·43±0·96	8·22±0·91	8·10±0·98	8·50±1·16
Hb	12·9 ±1·18	13·2 ±0·97	13·2 ±1·44	13·0 ±1·65

acetoacetate (0·3%) in the diet did not check the fall in erythrocyte count and haemoglobin appreciably, although the hydrolysed product of glucose cycloacetoacetate was almost as efficacious as vitamin B_{12} in maintaining the erythrocyte count and haemoglobin level. The meagre haematopoietic activity of glucose cycloacetoacetate as compared with its hydrolytic product may be attributed mostly to its poor absorption through the gastrointestinal tract because of the oral administration. Antianaemic behaviour of the hydrolysed product of glucose cycloacetoacetate and perhaps also of vitamin B_{12} may be, broadly, due to the protection offered by these substances against liver damage resulting from necrogenic diet. During the first phase of the development of dietary liver necrosis, depletion of glutathione occurs (Lindon & Work, 1953) and both vitamin B_{12} and hydrolysed product of glucose cycloacetoacetate have been found to check the depletion of glutathione (unpublished data). Further, the hydrolysed product of glucose cycloacetoacetate also facilitates the biosynthesis of methionine in rabbits in experimental atherosclerosis (Nath & Saikia, 1959). Methionine, being the precursor of cystine, checks the glutathione depletion in dietary liver necrosis (Leaf & Neuberger, 1947). The turnover of labelled haemoglobin formed from injected radioactive glycine is also increased in animals supplied with additional methionine in the diet (Cohen & Berg, 1956).

SUMMARY

1. The fall in the erythrocyte count and haemoglobin level in rats during 5 weeks of feeding necrogenic diet and the effect of glucose cycloacetoacetate, its hydrolysed product and vitamin B_{12} in restoring the haemoglobin level and erythrocyte count was studied.

2. The erythrocyte and haemoglobin levels in control animals kept on a necrogenic diet were lowered to 64 and 66% of the average values for normal animals respectively, whereas, in the animals kept on a necrogenic diet but receiving injections either of vitamin B_{12} or of hydrolysed glucose cycloacetoacetate, no significant fall in erythrocyte count and haemoglobin level was observed.

3. The nature of the antianaemic effect of vitamin B_{12} and hydrolysed product of glucose cycloacetoacetate has been discussed.

The authors record their grateful thanks to the Indian Council of Medical Research whose Research Grant supported this work.

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Biochem. J. (1961) **78**, 745

The Lipotropic Action of some Halogen Derivatives of Acetic Acid

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(*Received* 3 *August* 1960)

In previous work it was found that ethyl trichloroacetate was effective in lowering the high amount of liver lipid which accumulated when rats were fed a choline-deficient diet (Kratzing & Windrum, 1957, 1959). It seemed likely that other halogen derivatives of acetic acid might also possess this lipotropic action.

The investigation reported here shows that ethyl trifluoroacetate was able to prevent accumulation of excess of lipids in the liver of choline-deficient rats. Ethyl acetate, ethyl dichloroacetate and ethyl tribromoacetate were without demonstrable lipotropic action.

METHODS

Animals. The present experiments were carried out in separate laboratories; two different locally bred strains of young rats were used. The animals were housed separately in wire cages with wire-mesh floors. Strain 'A' rats were from the Physiology Department colony and were the same strain used for the experiments described in the initial papers (Kratzing & Windrum, 1957, 1959). Strain 'B' rats were from the Brisbane Hospital colony and had been used for experiments described in another paper (Windrum & Kratzing, 1960).

Diet. Strain 'A' rats were fed *ad lib.* on the diet used in previous experiments (Kratzing & Windrum, 1959). Strain 'B' rats were fed on the same diet to which 0·4% of cystine was added in order to increase the requirement of choline as discussed previously (Windrum & Kratzing, 1960). The amount of food fed to strain 'B' rats was adjusted to maintain the growth of all groups at an approximately uniform rate. Choline was fed to the control groups as choline chloride.

Esters. Ethyl trichloroacetate was prepared as described by Kratzing & Windrum (1959). Ethyl tribromoacetate, ethyl trifluoroacetate and ethyl dichloroacetate were prepared from the corresponding acids and the fractions which boiled between 120° and 125° (at 35 mm. Hg), 62° and 64°, and 156° and 159° respectively were used for injection. Ethyl acetate was a redistilled commercial sample.

Treatment. The esters were given for a 3- or 4-week period from the time of commencement of the deficient diet, except that strain 'A' rats, given ethyl dichloroacetate, were fed the choline-deficient diet for 3 weeks before commencing injections. The animals were injected subcutaneously once daily 5 days a week. Series 'B' rats were given the ethyl acetate and ethyl trichloroacetate as a 1:1 dilution in liquid paraffin for 4 weeks. Ethyl trifluoroacetate was injected as such because it was not completely miscible with paraffin. In order to minimize any lipotropic effect of the injection itself (see Kratzing & Windrum, 1959), strain 'A' choline-deficient control rats received injections of 0·9% sodium chloride and strain 'B' animals were given subcutaneous injections of liquid paraffin.

The animals were killed 24 hr. after the last injection and exsanguinated, and the livers and kidneys were excised and weighed.

Analysis. The liver-lipid content of strain 'A' rats was determined by the method described by Wheeldon & Collins (1957). The lipid content of strain 'B' rat liver was determined by the method used by Windrum & Kratzing (1960).

RESULTS

Ethyl acetate. Expt. 1 of Table 1 shows that strain 'A' rats fed on the choline-deficient diet did not respond with lowered lipid content of the liver when given repeated doses of ethyl acetate. The dosage given was that previously found effective with ethyl trichloroacetate (on an equimolar basis) in reducing excess of liver lipids (Kratzing & Windrum, 1959).

In this experiment the administration of either ethyl acetate or trichloroacetate did not result in any significant change in the rate of growth of animals in the different groups. The ratio of liver weight to body weight was significantly ($P < 0.01$) greater in choline-deficient and ethyl acetate-treated rats than in the choline-fed controls. It was also significantly greater in the ethyl trichloroacetate-treated group. The ratio of kidney weight to body weight was significantly increased in the ethyl trichloroacetate-supplemented groups compared with the choline-deficient group ($P < 0.01$). The choline- deficient group had a kidney to body weight ratio that did not differ significantly from the choline-supplemented group. However, the ethyl acetate group had a kidney to body weight ratio that was significantly ($P < 0.001$) higher than the comparable choline-deficient controls.

In a confirmatory experiment (Expt. 3, Table 1) in which rats with established choline deficiency were given 2–3 times the previous dose of either ethyl acetate or trichloroacetate, only the latter was effective in clearing the liver of excess of lipid.

Strain 'B' rats also did not respond to ethyl acetate supplements. A group which had received 20 doses of 360 μmoles of ethyl acetate in paraffin during 28 days on the deficient diet had a mean liver-lipid content of 15%, which was not significantly different from that of the paraffin-injected controls (Table 2).

Ethyl tribromoacetate. This was not as effective as ethyl trichloroacetate in removing excess of lipid

Table 1. *Effect of halogen-containing esters on organ weights and liver-lipid content*

Strain 'A' rats while on a choline-deficient diet were given the following esters by subcutaneous injection. Choline, when added, was given at 20 mg./10 g. of diet. The rats in Expts. 1 and 4 were all males. In Expts. 2 and 3 the sexes were mixed. Figures represent the average values ±S.E.M. Abbreviations: EDCA, ethyl dichloroacetate; ETCA, ethyl trichloroacetate; ETFA, ethyl trifluoroacetate; ETBA, ethyl tribromoacetate; EA, ethyl acetate. Choline: +, present; −, absent.

Expt. no.	Choline	Ester	No of rats	Dosage of ester (μmoles)	Initial wt. (g.)	Final wt. (g.)	Kidney wt. as % of body wt.	Liver wt. as % of body wt.	Lipid content of wet liver (%)
1	−	.	10	.	70±1	96±4	0·96±0·02	4·68±0·21	13·6±2·5
	−	EDCA	10	360	70±1	90±2	1·11±0·02	4·93±0·12	6·6±0·6
	−	ETCA	10	540	70±1	93±3	1·05±0·02	5·19±0·12	5·9±0·3
	−	EA	10	720	70±1	92±2	1·08±0·04	5·42±0·35	12·9±1·5
	+	.	10	.	70±1	97±3	0·94±0·02	4·01±0·11	4·9±0·2
2	−	.	10	.	73±3	89±4	0·93±0·02	5·65±0·18	23·9±2·3
	−	ETCA	10	360	72±3	86±6*	0·91±0·02	4·98±0·14	8·0±0·9
	−	ETFA	10	360	73±2	80±4	1·09±0·04	5·52±0·15	6·7±0·3
	+	.	10	.	73±3	89±4	0·87±0·03	3·80±0·12	4·8±0·2
3	−	.	8	.	68±4	87±4	.	.	11·7±1·5
	−	EA	7	1440	69±4	72±8†	.	.	9·1±1·4
	−	ETCA	6	1440	66±4	86±9†	.	.	5·0±0·1
4	−	.	9	.	74±3	106±3	1·11±0·05	5·50±0·28	16·9±2·0
	−	ETBA	10	520	85±3	86±3†	1·29±0·07	6·71±0·32	7·8±1·0
	+	.	10	.	84±3	118±3	0·93±0·02	4·27±0·12	3·5±0·3

* Two male rats died in this group.
† One rat died in this group.

Table 2. *Effects of halogen-containing esters on liver weight and lipid content*

Strain 'B' rats received the following halogen-containing esters while on a choline-deficient diet. All groups were of mixed sexes. Choline-deficient control groups received subcutaneous injections of paraffin. Choline, when added, was given at 10 mg./10 g. of diet. Abbreviations are as in Table 1.

Choline	Ester	No. of rats	Dosage of ester (μmoles)	Mean rat wt. Initial (g.)	Mean rat wt. Final (g.)	Liver wt. as % of body wt.	Lipid content in wet liver (%)
−	.	10	.	52	71	4·58	17·7
−	ETCA	10	220	53	68*	5·06	7·3*
−	ETFA	10	360	51	70*	5·52	6·1*
−	EA	10	360	58	77	4·46	15·0
+	.	8	.	65	90	3·29	4·2

* Two rats died in this group.

from the liver of choline-deficient rats (Table 1). There was a significant fall in the total lipid content of the liver when ethyl tribromoacetate was given to choline-deficient rats, but the lipid content was still significantly ($P = 0{\cdot}001$) higher than that of rats given choline. At the dosage of ethyl tribromoacetate given, the rats appeared listless and failed to gain weight. There was a significant increase in both kidney and liver weights when expressed as a ratio to body weight ($P < 0{\cdot}05$ and $P < 0{\cdot}02$ respectively).

Ethyl trifluoroacetate. In Expt. 2 (Table 1) it was found that this was as effective as ethyl trichloroacetate in lowering excess of liver lipids in choline-deficient rats. In addition, ethyl trifluoroacetate-treated rats had significantly increased ratios of both kidney and liver weights when expressed as a ratio of body weight. It was noticeable that at the site of injection there was more tissue breakdown with ethyl trifluoro- than with ethyl trichloro-acetate.

The 'B' strain of rats also responded to ethyl trifluoroacetate supplements by having a liver-lipid content significantly lower than the paraffin-injected controls (Table 2).

The addition of choline to the diet was more effective in reducing the lipid content of the liver than was either the trichloro or the trifluoro compound.

Ethyl dichloroacetate. This, when given to rats with established choline deficiency, was more toxic than an equimolar amount of ethyl trichloroacetate. With strain 'A' rats (four males and three females of approx. 65 g. body wt.) one dose of 1440 μmoles of ethyl dichloroacetate/100 g. produced a moribund condition in all seven in less than 24 hr. The mean liver-lipid weight per g. of wet liver for the group was 15·9 %.

Eight female rats of similar body weight received five daily doses of 1440 μmoles of ethyl dichloroacetate/100 g./day. These animals were fed on the rat-colony breeding ration throughout the period of injection. All animals survived with slightly increased body weight.

Eight strain 'B' rats received a dosage of 360 μmoles of ethyl dichloroacetate/100 g./day for 5 days per week during the administration of the choline-deficient diet. Seven animals died during the second and third week, and one animal, which survived for 26 days, had a liver-lipid content of 21 %.

DISCUSSION

In previous papers (Kratzing & Windrum, 1957, 1959) it was reported that ethyl trichloroacetate was able to decrease excess of liver-lipid content when given to choline-deficient rats. The present experiments confirmed earlier results and showed that ethyl trichloroacetate consistently and significantly lowered the concentration of liver lipids of the choline-deficient rats.

Ethyl trifluoroacetate was now found to act as a lipotropic factor and appeared to lower the concentration of liver lipids as effectively as ethyl trichloroacetate. On the other hand, neither ethyl acetate nor ethyl dichloroacetate showed any lipotropic effect at comparable dosage.

Ethyl tribromoacetate injections decreased the lipid content of the liver but the treatment stopped the growth of the rats. The relationship between growth and choline requirement of rats has been established (Best, Lucas & Ridout, 1954). In addition, Hartroft (1954) has stated that rats which become sick and listless will be able to mobilize much of the excess of lipid in the liver to produce 'the pseudolipotropic effect of caloric restriction' (Best, Hartroft & Sellers, 1952; see also Best & Ridout, 1938). It is likely that for ethyl tribromoacetate the lowering of the lipid content was due to a failure to grow and consequently these experiments do not indicate whether it is a true lipotropic factor or not.

The choline-deficient groups had consistently heavier livers than the choline-supplemented groups and this appeared to be related to the higher lipid content of the former. The esters produced liver weights which were comparable with the choline-deficient groups, and this effect was independent of their lipotropic effect as it was produced by ethyl acetate as well as by the trichloro and trifluoro compounds.

The relatively heavier kidneys produced by all the esters, whether lipotropic or not, suggest that they increased renal damage originally produced by the choline deficiency (Griffith, 1940; Moore, 1957). Previous work has suggested that ethyl trichloroacetate did not prevent renal degeneration in rats on a choline-deficient diet (Windrum & Kratzing, 1960).

With experiments of the type described in this paper, changes in metabolism are more likely to be recognized if the causative agent can exert its effects over long periods of time. For this reason, the ester forms of the halogen acids were chosen rather than the water-soluble salts, which are likely to be more rapidly excreted from the body. In addition, administration of the esters avoided the difficulties involved in injecting large amounts of water-soluble material.

Because of the similarity in chemical structure between ethyl trichloro- and trifluoro-acetate, it is likely that both compounds exert their effect in a similar way. The large energy of the C·F bond makes it unlikely that ethyl trifluoroacetate has lipotropic action as a result of any loss of fluorine atoms. Similarly, it is most probable that ethyl trichloroacetate does not lose chlorine atoms in the animal

body but rather that both ethyl trichloro- and trifluoro-acetate are effective as lipotropic agents because of the CCl_3 or CF_3 group. These groups are larger than the CH_3 group and may exert their effects through a steric blockage of an enzyme system.

Ethyl dichloroacetate was not only without lipotropic action but was highly toxic when given to rats in an established choline-deficient state. Rats fed on a ration which supported the breeding colony tolerated the same dosage with no noticeable impairment of function. It is probable that it is more toxic when the quality of protein in the diet is poor since it was also toxic when choline was added to the experimental diet (Kratzing, 1960). Laug (1946) stated that dichloroacetate was a more effective inhibitor of mouse-liver respiration than corresponding concentrations of trichloroacetate, whereas acetate, at the same concentration range, was not inhibitory. But Woodard, Lange, Nelson & Calvery (1941) found that rats tolerated larger oral doses of dichloroacetic acid than they did of trichloroacetic acid or acetic acid before showing signs of toxicity. In general the non-toxicity of the polyhalogen derivatives of acetic acid is in marked contrast to the toxicity of the monohalogen derivatives (Kratzing, 1960).

SUMMARY

1. Ethyl trifluoroacetate appeared as effective as ethyl trichloroacetate in decreasing excess of liver-lipid content of choline-deficient rats; whereas ethyl acetate and ethyl dichloroacetate were without effect.

2. Because of failure of the rats to grow, no lipotropic properties could be attributed to ethyl tribromoacetate.

We would like to express our appreciation for the technical assistance of Mrs G. Dabelstein and Miss M. Muir in this investigation. This work was supported by grants from the Commonwealth Research Fund and the N.H. and M.R.C. of Australia.

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Biochem. J. (1961) **78**, 748

Studies in the Biosynthesis of Fungal Metabolites

2. THE BIOSYNTHESIS OF ALTERNARIOL AND ITS RELATION TO OTHER FUNGAL PHENOLS*

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(*Received* 20 *July* 1960)

The mode of biosynthesis of certain types of fungal phenols involves the condensation of acetate units (for a review see Birch, 1957). However, the biosynthesis of alternariol, $C_{14}H_{10}O_5$ (I), a metabolite of *Alternaria tenuis* auct. (Raistrick, Stickings & Thomas, 1953), was of particular interest since, in addition to its rarity among known fungal products as a diphenyl derivative, it appeared to constitute a classical example of the acetate hypothesis. Thus it can in theory be derived solely by the head-to-tail condensation of seven acetate units without requiring additional stages involving oxidation, reduction, decarboxylation, etc. Another example of a fungal phenol, exclusively derived from acetate, is the key substance orsellinic acid, $C_8H_8O_4$ (II), isolated from *Chaeto-*

* Part 1: Chaplen & Thomas (1960).

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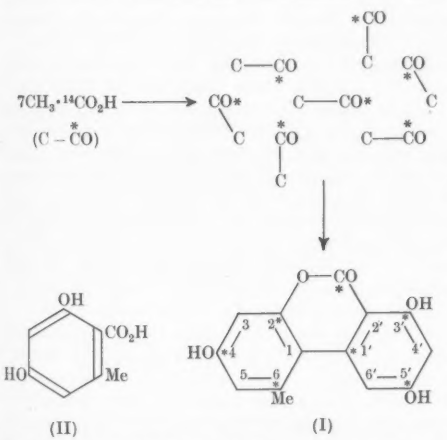
mium cochliodes (Gatenbeck & Mosbach, 1959) and *Penicillium madriti* (J. H. Birkinshaw & A. Gowlland, 1959, personal communication). The origin of alternariol is also of fundamental importance in relation to several other phenolic products of *A. tenuis* (Rosett, Sankhala, Stickings, Taylor & Thomas, 1957). The validity of this biosynthetic pathway (Fig. 1) was examined through a study of the mode of incorporation of sodium [1-^{14}C]acetate. Thus in theory every alternate carbon atom in alternariol should acquire approximately the same labelling. The determination of the resulting distribution of active C atoms strongly supported the above mechanism, as was described in a preliminary account by Thomas (1959).

In order to determine the conditions conducive to an efficient uptake of labelled acetate, the rate of production of alternariol was studied over a period of 77 days. A strain of *A. tenuis*, L.S.H.T.M. catalogue number 108 (Rosett *et al.* 1957), was selected which produces alternariol together with its monomethyl ether in yields of up to 13 % of the dry weight of the mycelium. The mould was grown at 24° on Czapek–Dox solution in surface culture and extracted as described by Raistrick *et al.* (1953). The results obtained from combined batches of four flasks removed at 7-day intervals are shown in Fig. 2.

The complete utilization of glucose (about 49 days as estimated polarimetrically) coincided with the maximum yield of the mycelium, which then remained essentially constant up to 70 days. The light-petroleum extract of the mycelium (lipid fraction) also reached its upper limit at 49 days, but subsequently declined fairly rapidly. As illustrated in Fig. 2, the individual glucose and mycelium values did not deviate significantly from the smooth curves shown, and even the lipid fraction (with the exception of the 28-day value) increased continuously up to its peak at 49 days. However, the ether extract, consisting mostly of alternariol and its monomethyl ether, progressed to a maximum (about 56 days) in a highly erratic manner. This probably reflects a considerable variation in the production of this fraction between individual flasks, relative to the total mycelium and its lipid fraction. The apparent brief continuation of alternariol production beyond 49 days at the expense of the lipid fraction, if significant, could be simply interpreted as resulting from the reassembly of acetyl-coenzyme A units, assumed to arise during the ensuing metabolism of fatty acids.



(II) (I)

Fig. 1. Suggested pathway of alternariol biosynthesis.

Labelled acetate was added to the growing culture after 19 days and the mycelium harvested at 29 days. These times were selected to fall within the phase of maximum rates of growth and alternariol production, as it was considered that a delay in harvesting until the optimum yield time (about 56 days) might increase any risk of interconversion of the C atoms of acetate. Incorporation of acetate readily took place and the lipid and alternariol fractions possessed very similar activities (about 13 μc/g.) in keeping with a common acetate origin. Under these conditions, approx. 6 % of the added labelled acetate was converted into alternariol and its monomethyl ether.

In strain 108 of *A. tenuis*, alternariol methyl ether was present in the ether extract as the minor component, and its complete separation from alternariol proved to be uneconomical. Consequently, on the assumption that they both arise through a common biosynthetic pathway, the mixture was converted into alternariol by demethylation with hydriodic acid before the distribution of labelling was determined. The effluent methyl iodide was combined with dimethylaniline to form phenyltrimethylammonium iodide which was devoid of radioactivity, thus demonstrating that the methoxyl carbon does not arise in this organism from acetate carboxyl. This agrees with the similar findings of Birch, Massey-Westropp, Rickards & Smith (1958*c*) in their study of the biosynthesis of griseofulvin, a product of *Penicillium griseofulvum*, although with aurantiogliocladin from *Gliocladium roseum* this interconversion apparently does take place to a small extent (Birch, Fryer & Smith, 1958*b*).

The crude labelled alternariol was converted into its trimethyl ether $C_{17}H_{16}O_5$ (III), which was recrystallized from acetone and diluted tenfold with unlabelled material. It was then systematically degraded as shown in Fig. 3, many of the stages being based on the previously described chemistry of alternariol (Raistrick *et al.* 1953).

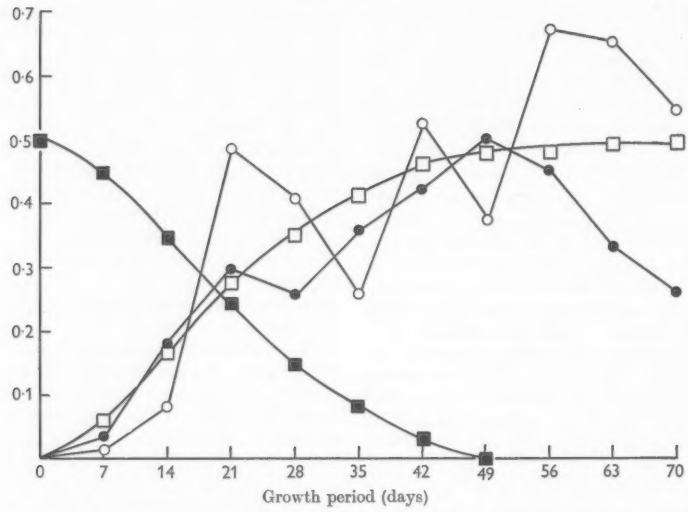


Fig. 2. Growth curves for *A. tenuis*. Average results from four combined flasks. ■, 10^{-1} × glucose (%); □, 10^{-1} × mycelial weight (g.); ○, ether extract (g.) (crude alternariol); ●, petroleum extract (g.).

Table 1 lists the degradation products whose activities were determined in order to calculate the labelling of the individual alternariol carbon atoms.

A Kuhn–Roth oxidation yielded acetic acid containing the methyl carbon and carbon atom 6 of alternariol (I). This was partially converted into its *p*-bromophenacyl ester, and the remainder was further degraded by pyrolysis of the lithium salt to lithium carbonate and acetone, which was then converted into iodoform with alkaline iodine (Cornforth, Hunter & Popják, 1953). The iodoform in turn was oxidized to carbon dioxide and assayed as barium carbonate.

Further methylation of alternariol trimethyl ether (III) under aqueous alkaline conditions produced the ester $C_{19}H_{22}O_6$ (IV). This was demethylated and decarboxylated by refluxing with a mixture of red phosphorus and hydriodic acid, yielding the tetrahydroxymethyldiphenyl (V); the effluent carbon dioxide corresponded to the lactone carbonyl of alternariol.

An unsuccessful attempt to prepare a pentanitro derivative of (V) by the action of a mixture of sulphuric acid and nitric acid gave rise instead to a product $C_{13}H_6O_{14}N_4S$, which was interpreted as resulting from the introduction of four nitro groups and the formation of a δ-sultone as shown in the structure (VI). Cullinane, Morgan & Plummer (1937) obtained the corresponding sultone of 2-hydroxydiphenyl-2′-sulphonic acid on refluxing *o*-phenylphenol with 70 % sulphuric acid. It was considered that the stability of the δ-sultone ring was responsible for the failure of a nitro group to enter position 2′. The product (VI) was then oxidized with barium hypobromite, and the resulting bromopicrin separated by steam distillation, converted by wet combustion into carbon dioxide and assayed as barium carbonate. The absence of any activity was consistent with the derivation of the four nitro C atoms 3, 5, 4′ and 6′ from the methyl C atom of acetate. However, only 58 % of the theoretical yield could be obtained, whereas a parallel oxidation of trinitro-orcinol yielded bromopicrin equivalent to 85 % of theory. Consequently it is possible that the bromopicrin was derived from only three of the four nitro C atoms present in the sultone (VI), although in view of the assigned structure it seems improbable that any one group would be completely resistant to attack. Assuming that all four nitro C atoms do in fact contribute to the bromopicrin and are consequently devoid of radioactivity, then it follows that C-2′ in alternariol is also inactive, since it is indistinguishable from C-6′ in the tetrahydroxymethyldiphenyl (V) from which the sultone was derived.

Alkaline permanganate oxidation of alternariol trimethyl ether (III) yielded a mixture of 3:5-dimethoxyphthalic acid and 4:6-dimethoxyphthalonic acid (VII) (Raistrick *et al.* 1953) containing ring B of alternariol. The latter product was refluxed with hydriodic acid in the presence of red

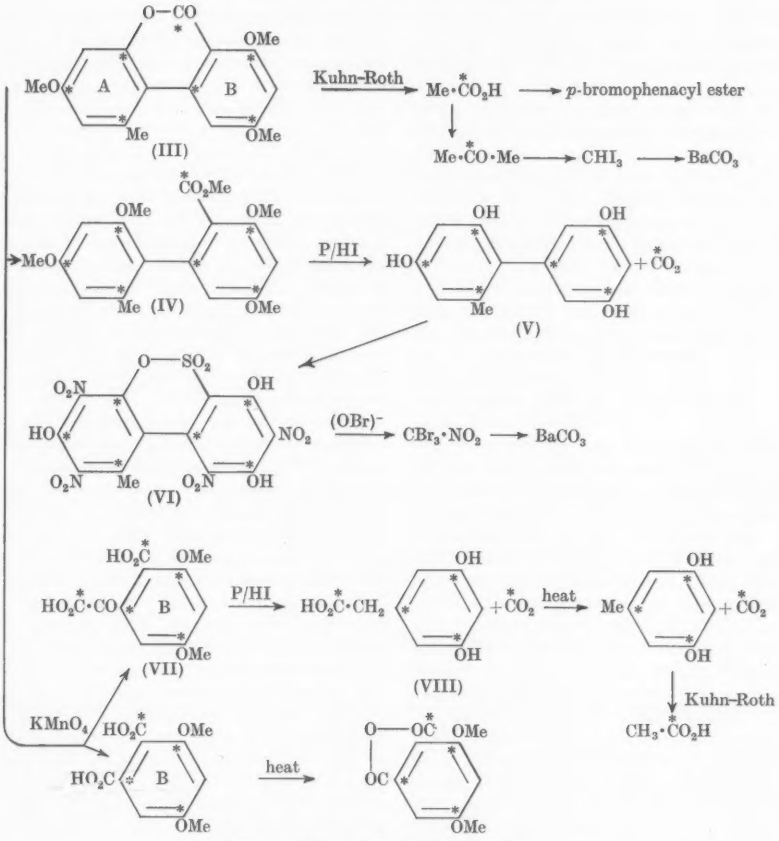


Fig. 3. Alternariol degradation scheme.

Table 1. *Activities of alternariol degradation products*

Degradation product	Alternariol derived C atoms	Activity (μc/mole) Found	Theory*
Alternariol trimethyl ether	1–6, 1′–6′, CO, Me	460	7 × 65·7
p-Bromophenacyl acetate	6, Me	59·1	65·7
p-Bromophenacyl acetate	1, 1′	59·4	65·7
$BaCO_3$ (via IV)	CO	74·7	65·7
$BaCO_3$ (via VII)	CO	74·7	65·7
$BaCO_3$	Me	0	0
$BaCO_3$	2	69·1	65·7
$BaCO_3$ (ex bromopicrin)	3, 5, 2′, 4′, 6′	0	0
$BaCO_3$	1	0	0
3:5-Dimethoxyphthalic anhydride	1, 1′–6′, CO	259	263
Orcinol	1, 1′–6′	184	197
2:3′:4:5′-Tetramethoxy-6-methyldiphenyl-2′-carboxylic ester	1–6, 1′–6′, CO, Me	454	460
2:3′:4:5′-Tetrahydroxy-6-methyldiphenyl	1–6, 1′–6′, Me	385	394
4:6-Dimethoxyphthalonic acid	1, 2, 1′–6′, CO	328	328
3:5-Dihydroxyphenylacetic acid	1, 2, 1′–6′	247	263

* Based on a theoretical incorporation of seven acetate units.

phosphorus when demethylation, reduction and decarboxylation took place giving rise to 3:5-dihydroxyphenylacetic acid (VIII). The carbon dioxide evolved again corresponded to the lactone carbonyl of alternariol and its activity when assayed as barium carbonate was in good agreement with that previously obtained from the ester (IV). Decarboxylation of the acid (VIII) yielded orcinol and carbon dioxide derived from C-2. Finally, a Kuhn–Roth oxidation of orcinol produced acetic acid which was degraded as before to determine the labelling of carbon atoms 1 and 1′.

In conjunction with the previous results, a knowledge of the activity of the phthalonic acid permitted the calculation by difference of the activity of C-4. However, the hydration of the phthalonic acid (which analysed as $C_{11}H_{10}O_6,H_2O$ even after vacuum drying at 100°) made it an equivocal standard. A more reliable assessment of the combined activities of its constituent carbon atoms was computed from the sum of the specific activities of 3:5-dimethoxyphthalic anhydride, prepared by dehydration of the free acid and barium carbonate, derived from C-2 in the course of decarboxylation of 3:5-dihydroxyphenylacetic acid. However, this alternative evaluation proved to be almost identical with the observed activity of the phthalonic acid monohydrate. The average activity of carbon atoms 3′ and 5′ was calculated from the known values of carbons 1, 1′, 2′, 4′, 6′ and orcinol.

The observed distribution of labelling (Table 2) clearly substantiated the postulated pathway involving acetate, thereby justifying the classification of alternariol with a number of other fungal phenols of similar biosynthetic origin. Some slight variation in the activities of alternate carbon atoms was apparent which may be significant. In particular the contrast between the lactone carbonyl and C-6 was outside the normal limits of experimental error; however, at present no obvious explanation of these results has been found.

The nature of the post-acetate intermediates is unknown, but if a sequential condensation of acetate units is assumed, then a consideration of the various alternative pathways leads to some interesting possible interrelationships. One mechanism of biosynthesis which has been suggested involves the oxidative coupling of orsellinic acid and 3:5-dihydroxyphthalic acid (Aghoramurthy & Seshadri, 1954). However, the orientation of hydroxyl substituents in alternariol is such that this would necessitate an abnormal type of phenol coupling (Barton & Cohen, 1957). The simpler direct pathway involving the condensation of seven acetate units (as in Fig. 1) requires carbon atoms 1 and 1′ to be derived from a single C_2 unit, thus precluding the independent origin of the two benzenoid rings. On this basis, orsellinic acid (arising from four acetate units, for example via IX) could in theory give rise to either but not both of rings A and B of alternariol (Fig. 4).

In order to test this possibility [*carboxy*-^{14}C]-orsellinic acid was synthesized from orcinol and sodium [^{14}C]cyanide and fed to *A. tenuis* under the conditions previously used for the uptake of acetate. However, the resulting alternariol was inactive, as it was also when orsellinic acid was added in the form of its ethyl ester with a view to overcoming possible disadvantages of the free acid, such as ease of decarboxylation or cell-wall-impermeability factors. This finding does not exclude the possibility of there being a C_{14} intermediate containing an intact six-membered ring, e.g. 'α-orsellinyl triacetic acid' (X), in which the orsellinic moiety corresponds to ring A of alternariol (Fig. 5). The particular theoretical advantage of this intermediate is that it can also give rise to a number of other natural phenols, as it is capable of various alternative modes of ring closure, including five (routes *A*–*E*) in which a second benzenoid ring is formed.

Thus biochemically feasible oxidation and reduction steps could lead to a product of structure (XI) which is a probable precursor of citromycetin (XII), a metabolite of *Penicillium frequentans* and other species, whose origin from acetate has been established by Birch *et al.* (1958*a*). It has been previously pointed out that the product (XI) could serve as a common precursor of citromycetin and fulvic acid (XIII) (involving oxygen-bridge formation), or alternatively it could yield the naphthalene derivatives fusarubin, purpurogenone and javanicin (probable structure XIV) following a carbocyclic condensation analogous to the route *A* folding of the intermediate (X) (Dean, Eade, Moubasher & Robertson, 1957; Whalley, 1958) as indicated in Fig. 5.

The complete derivation of javanicin from acetate in this manner is perhaps less certain than that of fusarubin and purpurogenone, as this would involve the reduction of the aromatic naphthalene

Table 2. *Distribution of labelling in alternariol derived from* [1-^{14}C]*acetic acid*

The relative activity is based on overall activity of seven units. Activities in parentheses were obtained by difference: activities of carbon atoms 3′ and 5′ represent the mean of their combined activities.

C atom	Relative activity	C atom	Relative activity
1	0	1′	0·90
2	1·05	2′	0
3	0	3′	(0·95)
4	(1·11)	4′	0
5	0	5′	(0·95)
6	0·90	6′	0
Me	0	CO	1·14

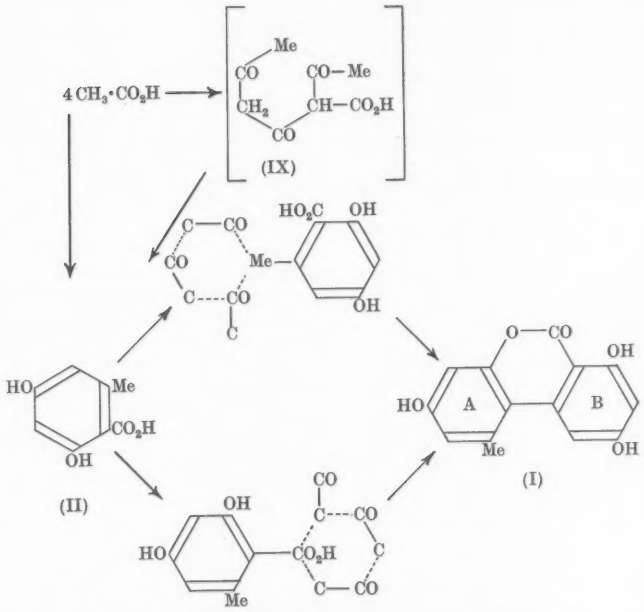


Fig. 4. Orsellinic acid as a possible alternariol precursor.

carboxyl to a methyl group, a process for which there does not at present appear to be a known analogy. Furthermore, as Birch *et al.* (1958*b*) showed that one of the methyl side chains of the dimethyl-substituted benzoquinone aurantiogliocladin arises by the replacement of an acetate carboxyl group by a C_1 unit (formate), one would expect that the corresponding methyl group of javanicin is derived from formate or some other C_1 donor.

This raises the interesting possibility that an analogous substitution of a polyisoprenoid side chain into an acetate-derived 3-carboxy-2-methyl-1:4-naphthaquinone by displacement of the carboxyl group could account for the biosynthesis of vitamins K_1 and K_2, in just the same way as Birch and co-workers have postulated a corresponding biosynthetic relationship between aurantiogliocladin and coenzyme Q.

An alternative carbocyclic condensation of the intermediate (X) is between C-atoms 1 and 6 (route *B*), yielding a benzophenone derivative, which could precede the formation of both the lichen product lichexanthone (XV) (Asahina & Nogami, 1942) and griseofulvin (XVI), another mould product of proven acetate origin (Birch *et al.* 1958*c*). On the other hand, a 6:7 condensation (route *C*) followed by δ-lactone formation would yield alternariol (I).

Although the product (XVII) of a 1:8 condensation (route *D*) has at present no known C_{14} analogue, it is closely related to the C_{15} plant phenol eleutherinol (XVIII) which occurs together with eleutherin (XIX) in *Eleutherine bulbosa* (Schmid, 1952). The carbon skeletons of both of these products could arise by the addition of a further acetate to the carboxyl of the acid (X) followed by decarboxylation and subsequent condensations analogous to routes *D* and *A* respectively. Furthermore, a route *B* type condensation of this C_{15} intermediate would yield a potential precursor of the fungal *p*-methylbenzophenone derivatives sulochrin (Nishikawa, 1939), geodin, erdin (Barton & Scott, 1958) and pinselin (Munekata, 1953), whereas in theory all three modes of folding (routes *A*, *B* and *D*) could precede formation of the polyhydroxy-2-methylanthraquinone group of mould pigments.

No examples of natural products related to the acid (XX) resulting from a 4:2′ condensation (route *E*) of (X) have been described. This may simply reflect the reluctance of a phenolic hydroxyl to undergo condensation, although the acid (X) need not represent the actual common precursor

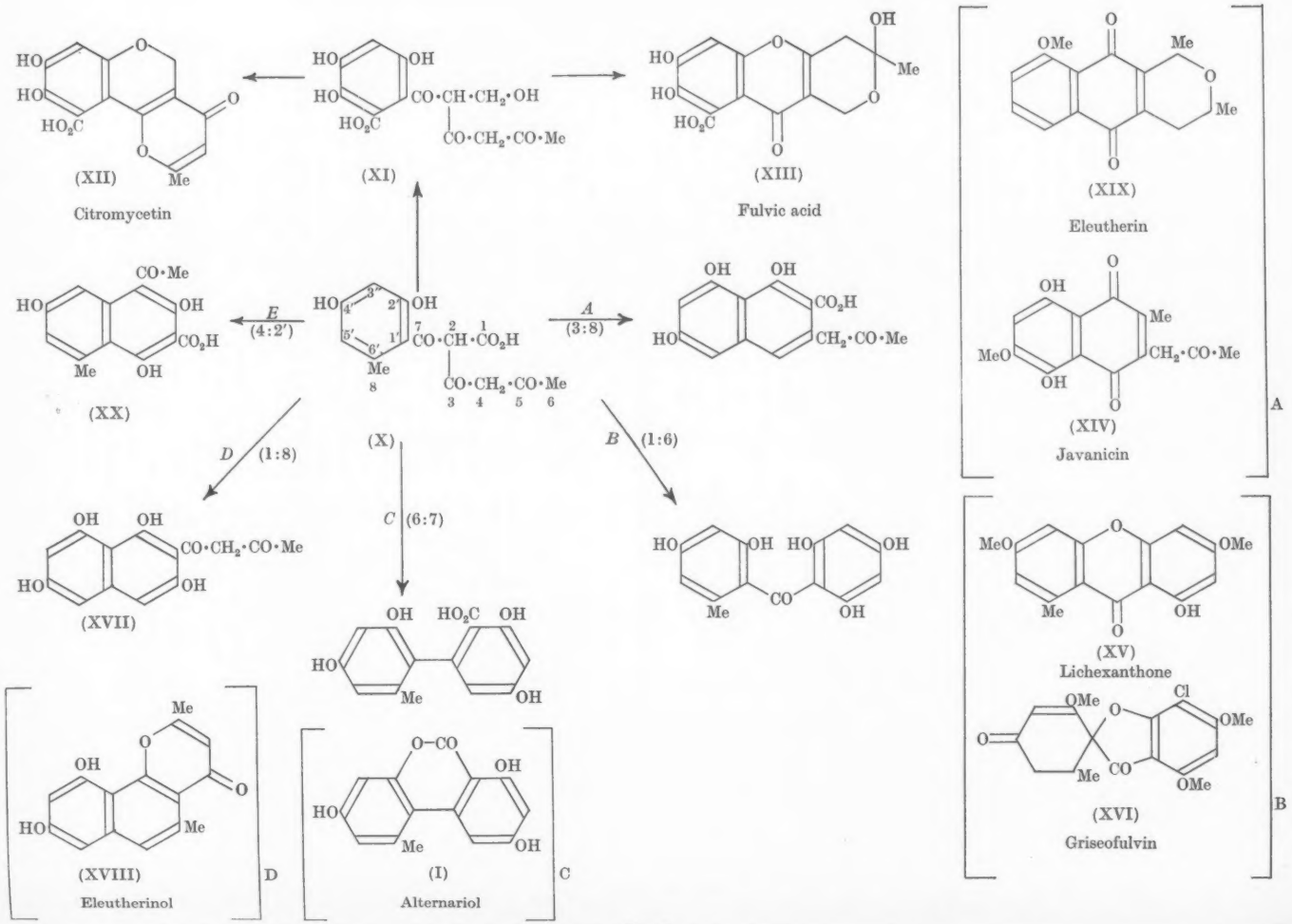

(XII)
Citromycetin
(XI)
(XIII)
Fulvic acid
(XIX)
Eleutherin
(XIV)
Javanicin
A
(XX)
E (4:2′)
A (3:8)
D (1:8)
(X)
C (6:7)
B (1:6)
(XVII)
(XV)
Lichexanthone
(XVIII)
Eleutherinol
D
(I)
Alternariol
C
(XVI)
Griseofulvin
B

Fig. 5

but only its carbon skeleton; thus the six-membered ring could still be present at a pre-aromatic stage.

In addition to the support given to this mechanism through the co-occurrence of eleutherin and eleutherinol, a further example of the possible use of alternative condensations of a common precursor exists in *Carpenteles brefeldianum*, which produces fulvic acid (XIII) on Raulin–Thom medium and griseofulvin (XVI) on Czapek–Dox (Dean *et al.* 1957).

If α-orsellinyl triacetic acid (X) does act as a common precursor of various bicyclic C_{14} fungal phenols, it follows that the branched side chain can itself serve as an aromatic precursor. Consequently if both rings of alternariol are formed in a similar manner, then the original ring [corresponding to orsellinic acid (II)] would arise from α-acetyltriacetic acid (IX) as illustrated in Fig. 4. This latter acid is the open-chain form of dehydracetic acid (XXI) (Fig. 6) and it is of interest that alternaric acid (XXII), a metabolite of *Alternaria solani* (Bartels-Keith & Grove, 1959) may be regarded as a dihydro-dehydroacetic acid derivative.

Dehydroacetic acid has been shown in *Penicillium urticae* to inhibit strongly the production of 6-methylsalicylic acid and gentisyl alcohol (Ehrensvärd, 1955). However, this could be explained as a consequence of competitive inhibition, since 6-methylsalicylic acid (being a deoxyorsellinic acid) would require in the above pathway the mediation of a dihydro derivative of dehydroacetic acid. It is perhaps significant that the particular dihydro-dehydroacetic acid required for 6-methylsalicylate synthesis is precisely that shown to be present in alternaric acid. Dehydroacetic acid could also ring-close to form acetophenone derivatives similar to phloracetophenone and clavatol (Hassall & Todd, 1947).

An alternative biosynthetic scheme involving the folding of linear poly-β-keto acid precursors has been elaborated by Birch (1957) and also by Woodward (1957), who in addition suggested the possible mediation of macrocyclic lactones of the type known to be present in the 'macrolide' class of antibiotics. On this basis the mould metabolite curvularin could be considered as an advanced intermediate, since it contains both a benzene and a macrocyclic lactone ring and has been shown to arise from acetate (Birch, Musgrave, Rickards & Smith, 1959).

However, although the linear polyacetate hypothesis could also explain the biosynthesis of alternariol, it is inadequate in cases such as citromycetin, fulvic acid, sulochrin, pinselin and geodin, which clearly require a branched-chain precursor.

EXPERIMENTAL

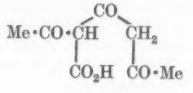
All melting points are uncorrected. Analyses for C, H, N and S were carried out by Weiler and Strauss of Oxford and by Mr F. H. Oliver of Parke, Davis and Co. Ltd., Hounslow, Middlesex.

Culture and medium. The culture of *Alternaria tenuis* was the London School of Hygiene and Tropical Medicine strain number S.M. 108, which was grown in surface culture on a Czapek–Dox medium as described by Raistrick *et al.* (1953).

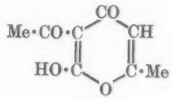
Radioactive assay. The ^{14}C content of samples mounted on 1·5 cm. diameter planchets was determined at infinite thickness with a thin end-window Geiger–Müller counter, as outlined by Chaplen & Thomas (1960).

Determination of alternariol production. Czapek–Dox medium (350 ml./flask) was inoculated with a mycelial suspension of *A. tenuis* in sterile water and incubated in the dark at 24°. Over 10 weeks, sample batches of four flasks were removed at regular intervals (4 days), and the combined culture filtrates examined for pH, residual glucose (determined polarimetrically) and ferric reaction. The combined mycelium was dried, weighed and extracted in a Soxhlet apparatus, initially with light petroleum (b.p. 40–60°) for 24 hr. and subsequently with dry ether for a further 24 hr. These extracts were evaporated to dryness; the ether extract was washed with boiling light petroleum before ultimately drying and weighing.

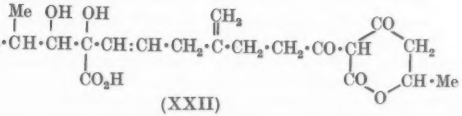
The pH of the culture filtrate rose from 4·40 to 7·65 in the first 2 weeks and increased only a further 0·2 unit in the following 5 weeks. The filtrate at 2 weeks and thereafter yielded a green colour on addition of a few drops of $FeCl_3$, which changed to a dark-brown solution accompanied by



(IX)



(XXI)



(XXII)

Fig. 6

a precipitate in the presence of an excess of the reagent. The other results are shown graphically in Fig. 2.

Incorporation of sodium [1-^{14}C]acetate. Autoclaved solutions of sodium [1-^{14}C]acetate (60 μc/5 ml.) were added to the metabolism liquors of 19-day cultures by careful pouring down the wall of each flask so as to pass under the mycelial pad. In this way, labelled acetate was added to ten flasks which were subsequently harvested after a total of 29 days' growth and extracted as above. The crude light-petroleum extract (2·60 g.) and the dry ether extract (2·72 g.) of the mycelium (36·06 g.) possessed closely similar activities (13 μc/g.). Thus the incorporation of added acetate into the light-petroleum extract (lipid fraction) and the ether extract (consisting largely of alternariol and its methyl ether) was in each case approx. 6%.

Demethylation of the ether extract. The ether extract (1·582 g.) was demethylated by refluxing with constant-b.p. HI (10 ml.) in the presence of phenol (4 g.) which facilitated solution of the alternariol methyl ether. The effluent methyl iodide was absorbed in pure dimethylaniline; the crystalline precipitate of phenyltrimethylammonium iodide which began to form after about 30 min., was filtered at 2 hr. and washed with chloroform. The dried crystals, m.p. 218°, when assayed for ^{14}C content were found to be devoid of radioactivity.

Demethylation of the ether extract was continued for a further 10 hr., after which time no further methyl iodide was evolved. The crude alternariol (1·452 g.) was recovered by addition of water and filtration of the resulting precipitate, which was washed thoroughly with hot water before drying.

Preparation and dilution of alternariol trimethyl ether. Crude alternariol (1·36 g.) was refluxed with dimethyl sulphate (6 ml.) and anhydrous K_2CO_3 (20 g.) in dry acetone (150 ml.) until the solution no longer gave a purple ferric reaction (8 hr.). It was then filtered and concentrated to yield alternariol trimethyl ether, $C_{17}H_{16}O_5$ (1·43 g.), which after crystallization from acetone melted at 161°. This product (1·003 g.) (activity, 4127 counts/min.) was diluted with pure inactive trimethyl ether (9·027 g.) and recrystallized from acetone (400 ml.), yielding the source material for the degradation studies whose activity (410 counts/min.$\equiv$460 μc/mole) exceeded the average background count rate approximately 50-fold.

Degradation of alternariol trimethyl ether. The preparation of many of the products used in these experiments has been described in detail by Raistrick *et al.* (1953) and where possible the same procedures were adopted.

Kuhn–Roth oxidation of alternariol trimethyl ether. Alternariol trimethyl ether (608 mg.) when oxidized by refluxing with a mixture of 5N-chromic acid (120 ml.) and conc. H_2SO_4 (30 ml.) produced a quantitative yield of acetic acid, which was separated by steam-distillation and neutralized with 0·1N-LiOH. The aqueous solution was concentrated by distillation and a portion converted into *p*-bromophenacyl acetate, which was purified by crystallization from light petroleum (b.p. 80–100°) and vacuum sublimation, yielding colourless needles, m.p. 84–85° (Found: activity, 59·1 μc/mole). The remaining lithium acetate was evaporated to dryness and pyrolysed in a current of N_2 at 390°; the effluent acetone was absorbed in water and converted into iodoform by treatment with aqueous alkaline iodine. The iodoform was oxidized to CO_2 and assayed as $BaCO_3$, which was found to be free from radioactivity.

Preparation of 2:3′:4:5′-tetrahydroxy-6-methyldiphenyl. Alternariol trimethyl ether (1·036 g.) was hydrolysed by gently refluxing with N-NaOH (20 ml.) for 15 min. The resulting solution was cooled, dimethyl sulphate (2 ml.) added and the mixture boiled for about a minute. This procedure gave rise to a crystalline deposit (1·166 g.) which was purified by crystallization from methanol, yielding the methyl ester of 2:3′:4:5′-tetramethoxy-6-methyldiphenyl-2′-carboxylic acid, m.p. 124° (Raistrick *et al.* 1953) (Found: activity, 454 μc/mole). A mixture of this ester (0·499 g.), red P (2 g.) and constant-b.p. HI (15 ml.) was heated in an oil bath at 140°, and the effluent CO_2 assayed as $BaCO_3$ (Found: activity, 74·7 μc/mole). The hydrolysis was continued for 2 hr. in order to obtain complete demethylation, after which the solution was filtered and cooled. The resulting crystalline precipitate (0·271 g.) was purified by sublimation in a high vacuum at 200°, yielding 2:3′:4:5′-tetrahydroxy-6-methyldiphenyl, m.p. 246–248° (Found: activity, 385 μc/mole).

Nitration of 2:3′:4:5′-tetrahydroxy-6-methyldiphenyl. 2:3′:4:5′-Tetrahydroxy-6-methyldiphenyl (0·59 g.) was heated at 100° with conc. H_2SO_4 (1·5 ml.) for 30 min. The mixture was cooled in an ice bath and conc. HNO_3 (4 ml.) slowly added, the temperature being maintained below 5° until the initial exothermic reaction was complete. The temperature was then raised to 70°, when a vigorous reaction took place giving rise to a yellow crystalline precipitate. When the reaction had subsided, the mixture was diluted with water (10 ml.) and cooled. After filtration and washing with ice-cold water, an initial crop of yellow microneedles was obtained (0·502 g.), m.p. 243°. A small second crop of needles (0·023 g.) separated from the filtrate on standing overnight. Ether extraction of the mother liquor from these two crops produced further material (0·240 g.).

The nitration product, after repeated crystallization from 0·1N-HCl, melted at 246° with vigorous decomposition. The analysis corresponded to that required for the δ-sultone of 2:3′:4:5′-tetrahydroxy-6-methyl-3:4′:5:6′-tetranitrodiphenyl-2′-sulphonic acid (Found: C, 32·6; H, 1·3; N, 11·4, 11·6; S, 7·0. $C_{13}H_6O_{14}N_4S$ requires C, 32·9; H, 1·3; N, 11·8; S, 6·8%).

Hypobromite degradation of the tetranitrosultone. The sultone (200 mg.) was dissolved in boiling water (10 ml.) and hot $Ba(OH)_2$ solution (20 ml.) added, giving rise to a reddish-brown precipitate. The mixture was then cooled to 4° and treated with a solution of Br_2 (0·5 ml.) in saturated $Ba(OH)_2$ (50 ml.) at the same temperature. The characteristic pungent odour of bromopicrin, $CBr_3{\cdot}NO_2$, was rapidly generated. The mixture was kept overnight and the product was collected by steam-distillation into a centrifuge tube. After repeated centrifuging and intermediate washing with water, the bromopicrin droplet was frozen and transferred to a flask for wet combustion to CO_2 under van Slyke–Folch conditions with chromic acid solution and subsequently precipitated as $BaCO_3$ (195 mg.$\equiv$58% of theoretical yield). The washed and dried product was devoid of any radioactivity.

Potassium permanganate oxidation of alternariol trimethyl ether. Alternariol trimethyl ether (3·90 g.) was refluxed with N-NaOH (65 ml.) for 15 min., after which the resulting solution was cooled and oxidized at room temperature with excess of aq. $KMnO_4$ (260 ml. of a 10% solution)

and kept overnight. The heavy MnO_2 precipitate was removed by filtration. The filtrate was adjusted to pH 7 with conc. HCl, and the excess of permanganate reduced by the addition of sodium dithionite. It was then acidified to pH 2 before extracting with ether (3 × 1 vol.). Evaporation of this extract yielded crystals of 3:5-dimethoxyphthalic acid (0·558 g.), m.p. 153–156°, and a partially crystalline residue (0·161 g.).

The ether-extracted oxidation solution was neutralized with 2N-NaOH and partially evaporated *in vacuo* to about 200 ml.; it was then reacidified and extracted continuously with ether for 24 hr. Concentration of this second ether extract yielded a largely crystalline pale-yellow gum (2·089 g.) which on repeated crystallization from water yielded 4:6-dimethoxyphthalonic acid, m.p. 173° (decomp.). Drying to constant weight at 100° in a high vacuum produced the acid monohydrate (Found: C, 48·1; H, 4·5; activity, 328 μc/mole. Calc. for $C_{11}H_{10}O_7,H_2O$; C, 48·5; H, 4·4%).

3:5-*Dimethoxyphthalic anhydride.* The $KMnO_4$ oxidation product, 3:5-dimethoxyphthalic acid, was purified by crystallization from water and dehydrated at 190° for 5 min., by which time the evolution of water was complete. On cooling, the melt crystallized and pure 3:5-dimethoxyphthalic anhydride was prepared by crystallization from *o*-xylene, followed by sublimation in a high vacuum, giving colourless needles, m.p. 148–149° (Found: activity, 259 μc/mole).

Reductive decarboxylation of 4:6-*dimethoxyphthalonic acid.* 4:6-Dimethoxyphthalonic acid (0·473 g.) was heated with a mixture of red P (1·0 g.) and constant-b.p. HI at 140° for a total of 8 hr. The effluent CO_2 was trapped as $BaCO_3$ which was recovered by filtration, washed with CO_2-free water, dried and assayed (Found: activity, 74·7 μc/mole). The reaction mixture was filtered while hot, cooled, and extracted with ether. Successive washing of the extract (150 ml.) with water (5 ml.) then conc. $Na_2S_2O_3$ solution (5 ml.) removed HI and I_2 respectively and on evaporation to dryness a partly crystalline colourless gum (0·231 g.) was obtained which completely crystallized on trituration with ether. The product (3:5-dihydroxyphenylacetic acid) was purified by vacuum sublimation at 130°, giving a crystalline sublimate, m.p. 130° (Found: activity 247 μc/mole).

Decarboxylation of 3:5-*dihydroxyphenylacetic acid.* 3:5-Dihydroxyphenylacetic acid (0·165 g.) was decarboxylated by heating the solid at 270° in a stream of N_2 gas; the effluent CO_2 was trapped as $BaCO_3$ (0·130 g.) (Found: activity, 69·1 μc/mole). The decarboxylation product was collected by sublimation on a 'cold finger' and then purified by further sublimation in a high vacuum at 70°. This yielded a crystalline sublimate (0·091 g.) of m.p. 107–108°, which was unchanged on admixture with orcinol purified by a similar sublimation procedure (Found: activity, 184 μc/mole).

Oxidation of the orcinol with CrO_3 under Kuhn–Roth conditions gave a quantitative yield of acetic acid, part of which was converted into its *p*-bromophenacyl ester as before (Found: activity, 59·4 μc/mole). The remainder was pyrolysed as lithium acetate to give acetone, subsequent alkaline-iodine treatment of which yielded iodoform as described above. When oxidized and converted into $BaCO_3$, this product proved to be inactive.

Synthesis of [carboxy-^{14}C]*orsellinic acid.* [*formyl*-^{14}C]-Orsellinic aldehyde was synthesized by a modification of the method of Adams & Levine (1923) in which 1 mc of $Na^{14}CN$ (9·8 mg.) was added to $Zn(CN)_2$ (3 g.) before reacting with orcinol (2·0 g.) in the appropriate manner. The resulting crude orsellinic aldehyde (2·16 g., m.p. 181°) was then converted into orsellinic acid as described by Hoesch (1913). This further sequence of reactions involved the initial formation of the diethoxycarbonyl derivative, m.p. 58°, followed by oxidation of the aldehyde with $KMnO_4$ in aqueous acetone to the acid, m.p. 115°, which was finally hydrolysed with dilute NaOH to orsellinic acid (1·50 g.). The free acid was purified by crystallization from water and sublimation in a high vacuum, yielding colourless needles, m.p. 184–185° (Found: activity, 72 μc/g.). The pure orsellinic acid, which melted at a higher temperature than the normally recorded value (176°), produced the expected purple ferric reaction in ethanolic solution. The identity of the product was confirmed by preparation of its methyl ester, m.p. 137° (recorded m.p. 138–140°), and also its ethyl ester, m.p. 130° (recorded m.p. 131–132°), by brief treatments of the acid with diazomethane and diazoethane respectively.

Attempted incorporation of orsellinic acid and ethyl orsellinate into alternariol. Approx. 7 μc of the above orsellinic acid (100 mg.) in ethanolic solution (2 ml.) was added to each of two flasks containing 18-day cultures of *A. tenuis*. One flask was harvested at 21 days and the other at 25 days. The mycelium was only slightly active (24 counts/min.) and the activities of both the light-petroleum and ether extracts of the mycelium were relatively negligible. A corresponding experiment with labelled ethyl orsellinate (3 μc in 50 mg.) in ethanol (2 ml.) also failed to produce significant incorporation of activity.

SUMMARY

1. The biosynthesis of alternariol, $C_{14}H_{10}O_5$, a phenolic product of *Alternaria tenuis*, has been studied.

2. Chemical degradation of labelled alternariol derived from sodium [1-^{14}C]acetate clearly demonstrated a biosynthetic mechanism involving the head-to-tail condensation of acetate units.

3. A possible common precursor with other fungal phenols containing C_{14} skeletons is discussed.

I am grateful to Imperial Chemical Industries Ltd. for the purchase of some of the tracer equipment and ^{14}C-labelled compounds used in this work, which was carried our during the tenure of an I.C.I. Research Fellowship. I also wish to thank Professor J. H. Birkinshaw for his continued interest and encouragement.

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Biochem. J. (1961) **78**, 758

The Metabolism of 3:5-Di-*tert.*-butyl-4-hydroxytoluene and 3:5-Di-*tert.*-butyl-4-hydroxybenzoic Acid in the Rabbit

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(*Received* 27 *April* 1960)

The compound known commercially as butylated hydroxytoluene (3:5-di-*tert.*-butyl-4-hydroxytoluene) is an antioxidant developed initially for use with petrol, petroleum products and rubber. Dugan, Marx, Weir & Kraybill (1954) found butylated hydroxytoluene to be an effective antioxidant for animal fats at a concentration of 0·01%, and legislative approval for its addition to fats and foods containing fats intended for human consumption has already been given in eight countries including U.S.A., Denmark, Sweden and the United Kingdom. The toxicity of the compound to rabbits, rats, guinea pigs, cats and dogs was investigated by Deichmann, Clemmer, Rakoczy & Bianchine (1955), who found that when it was given orally to rabbits, there was an increased excretion of both glucuronide and ethereal sulphate in the urine. The causes of the toxic and lethal effects in rabbits given large doses of butylated hydroxytoluene were investigated by Denz & Llaurado (1957) and some aspects of its chronic toxicity in rats were studied by Brown, Johnson & O'Halloran (1959).

It will be shown in this paper that 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid is a metabolite of butylated hydroxytoluene in both the free and conjugated forms. The metabolism of this acid and of the antioxidant butylated hydroxytoluene has been investigated in the rabbit and the major metabolites excreted in the urine have been isolated and characterized. A summary of these results has been published (Dacre, 1960).

EXPERIMENTAL

Animals, diet and dosage. The rabbits used were does of the New Zealand white strain, weighing 1·33–2·0 kg. They were maintained on a standard pellet diet (wheat, 40%; pollard, 33%; bran, 27%) and during experiments were kept singly in metabolism cages designed to permit the separate collection of urine and faeces. For the isolation of metabolites in quantity, rabbits were maintained on a modified diet (wheat, 35%; pollard, 20%; meat meal, 15%; bran, 10%; maize meal, 10%; milk powder, 7·5%; bone meal, 1·7%; cod-liver oil, 0·8%). Water and food were supplied *ad lib.* and the urine was collected daily and stored at −10°. In the initial experiments, single doses of 0·8 g. of butylated hydroxytoluene (BHT)/rabbit were dissolved in 7·2 ml. of olive oil and administered by stomach tube. In later experiments, doses of 0·5 and 1·0 g. in 5 ml. of olive oil/rabbit or 0·8 g. of BHT in 4 ml. of olive oil/kg. body wt. of the

animals were given. Three consecutive daily doses of 0·28 and 0·65 g. of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid in 5 and 10 ml. respectively of olive oil were similarly administered to a rabbit. The animals usually refused food during the 24 hr. after receiving single doses of BHT and they died between 4 and 10 days after the discontinuation of the repeated doses. No obvious ill-effects were observed in the rabbit receiving the three daily doses of the acid, apart from mild anorexia.

Materials. BHT (Ionol) was purchased from the Shell Company of New Zealand and bacterial β-glucuronidase from the Sigma Chemical Co., St Louis, Mo., U.S.A. 2:4-Dihydroxytoluene was given by Dr S. Dagley, University of Leeds; 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid and 3:5-di-*tert.*-butyl-4-hydroxybenzyl alcohol, for mixed m.p. and determination of spectra, were given by Dr G. R. Yohe, Section of Coal Chemistry, Illinois State Geological Survey Division, Urbana.

Preparation of reference compounds

Combustion analyses were carried out by Dr A. D. Campbell, Chemistry Department, University of Otago. All methoxy (OMe) values are corrected (see Campbell & Chettleburgh, 1959). All melting points are uncorrected.

Derivatives of butylated hydroxytoluene. 3:5-Di-*tert.*-butyl-4-hydroxybenzaldehyde was prepared by bromine oxidation of BHT dissolved in *tert.*-butanol according to Coppinger & Campbell (1953); 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid was prepared from the aldehyde by the Cannizzaro reaction (10–15% yield) and 3:5-di-*tert.*-butyl-4-hydroxybenzyl alcohol by lithium aluminium hydride reduction of the aldehyde according to Yohe *et al.* (1956). The acid was also prepared in greater yield from the urine of rabbits which had been given BHT orally.

3:5-Di-tert.-butyl-4-hydroxybenzoic acid from rabbit urine. Urine (250 ml., pH 5·65, containing approx. 16 μmoles of total phenols/ml.) from rabbits given BHT, was incubated with β-glucuronidase powder (0·2 g., about 10 000 units) with intermittent shaking in a water bath at 37° for 24–30 hr. The crystalline material which separated was collected, dried and extracted with peroxide-free ether (2 × 200 ml.) to remove the enzyme residue. Removal of the solvent *in vacuo* yielded 0·54 g. of colourless crystals, m.p. 185–195°. Extraction of the residual urine with peroxide-free ether (2 × 200 ml.) yielded a further 60 mg. (total recovery 64%). One recrystallization from light petroleum (b.p. 60–80°) or vacuum sublimation (110–120°/0·05 mm. Hg) gave colourless plates of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 210°, unchanged by admixture with an authentic sample (Found: C, 71·75; H, 8·9; mol. wt. 228. Calc. for $C_{15}H_{22}O_3$: C, 72·0; H, 8·8%; mol.wt. 250). Yohe *et al.* (1956) record m.p. 218° for this acid. When determined in sealed tubes from a cold electrically-heated block, Yohe's sample, the sample described above and a mixture of the two all melted at 209–210°.

Methyl 3:5-di-tert.-butyl-4-hydroxybenzoate. 3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid (100 mg.) was dissolved in dry ether (100 ml.) and diazomethane (about 2 g.) in dry ether was slowly added with intermittent shaking. The reaction mixture was kept for 4 hr. and the ether was then removed *in vacuo* to yield dull-yellowish crystalline plates of m.p. 163–165° (82 mg., 78%). Recrystallization from light petroleum (b.p. 60–80°) gave large colourless plates, m.p. 164–165° with sublimation, of *methyl 3:5-di-tert.-butyl-4-hydroxybenzoate* (Found: C, 72·75; H, 8·9; OMe, 11·2. $C_{16}H_{24}O_3$ requires C, 72·7; H, 9·1; OMe, 11·7%).

3:5-Di-tert.-butyl-2:4-dihydroxytoluene. 2:4-Dihydroxytoluene (1·5 g., m.p. 94–95°) was dissolved in dry benzene (50 ml.) and conc. H_2SO_4 (0·1 ml.) added. Dry *iso*butylene was bubbled through the stirred mixture at 50–60° for 4 hr. The benzene soln. was shaken out with 2N-NaOH (2 × 25 ml.) and then with water. The benzene was removed *in vacuo* to give almost colourless crystals (1·44 g., 50%) of m.p. 145–146°. Repeated sublimation *in vacuo* below 100° yielded large colourless rods, m.p. 146–147°, of *3:5-di-tert.-butyl-2:4-dihydroxytoluene* (Found: C, 76·4; H, 10·25. $C_{15}H_{24}O_2$ requires C, 76·2; H, 10·2%).

Analytical methods

All spectrophotometric measurements were made with a model SP. 600 spectrophotometer (Unicam Instruments Ltd., London).

Free and total phenols. Phenols were determined on diluted rabbit urine, both before and after acid hydrolysis, by the method of Folin & Ciocalteu (1927). The final concentration of phenol was adjusted to 0·3–1·5 mM and the developed colour was measured at 700 Å. The calibration curves of extinction against concentration for phenol, quinol, 2:4-dihydroxytoluene, 3:5-di-*tert.*-butyl-2:4-dihydroxytoluene and 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid gave the same line for the range 0·3–1·5 mM. BHT does not give a blue colour with the Folin & Ciocalteu reagent. When 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid (17·5, 35 and 70 μmoles/ml.) was added to urine, the mean recoveries, determined as free phenols, were 101·3, 98·9 and 90·0% respectively. This glucuronide therefore would contribute to free phenol values.

Non-conjugable phenols. These were determined by extracting urine at pH 5·5–6·5 with peroxide-free ether and analysing the evaporated extracts by the Folin & Ciocalteu method.

Glucuronides. (*a*) Total glucuronides. The naphtharesorcinol method, as modified by Bray, Humphris, Thorpe, White & Wood (1952), was used for the estimation of total glucosiduronic acid. The naphtharesorcinol reagent (aq. 0·4%) was prepared according to Fishman & Green (1955). Urine was diluted to give a glucuronide concentration between 0·5 and 2·0 mM and readings were made at 600 Å. A calibration curve was prepared with 2-*tert.*-butyl-4-methoxyphenyl β-D-glucosiduronic acid (Dacre, Denz & Kennedy, 1956).

(*b*) Ester glucuronides. The Shaffer & Hartmann (1921) copper reduction method as described by Bray & Thorpe (1954) was used for the estimation of glucosiduronic acid liberated by alkaline hydrolysis of ester-type glucuronides.

Hippuric acids. Glycine conjugates in diluted rabbit urine were estimated by the micro-method of Gaffney, Schreier, DiFerrante & Altman (1954) as modified by El Masry, Smith & Williams (1956). The yellow azlactone formed from the hippuric acid and *p*-dimethylamino benzaldehyde was measured at 460 Å. The calibration curve prepared with hippuric acid was linear over the range 0–0·56 mM.

Mercapturic acids. The method of Stekol (1936) (iodine consumption after hydrolysis by alkali) as modified by Parke & Williams (1951) was used.

Ethereal sulphates. These were determined by Sperber's (1948) modification of the turbidimetric method. The

calibration curve was made with K_2SO_4 and readings were taken in the range 0–1·0 mg. of SO_3.

Paper chromatography

Phenolic compounds and their metabolites were separated on Whatman no. 1 filter-paper sheets by the descending method with the solvent systems described in Tables 1 and 2. The solvent front was run for 30–40 cm. The compounds were detected and identified on paper with the following spray reagents (all prepared on a w/v basis). (1) Gibbs reagent: a solution of 2:6-dichloro-*p*-benzoquinone-4-chloroimine (0·1%) in ethanol (95%) followed by a solution of sodium tetraborate (aq. 2·0%, pH 9·24). (2) Pauly reagent: a freshly prepared diazotized sulphanilic acid soln. (0·3%) in HCl (8%) followed by a solution of Na_2CO_3 (aq. 20%). (3) Molybdophosphoric acid–ammonia reagent: a solution of dodecamolybdophosphoric acid (0·5%) in ethanol (95%) followed immediately by exposure to conc. ammonia fumes (Mitchell, 1957). (4) Naphtharesorcinol–trichloroacetic acid reagent: this was as described by Elliott, Parke & Williams (1959); the reagent does not react on paper with acetylated–methylated glucuronides. (5) Altman reagent: a freshly prepared solution of *p*-dimethylaminobenzaldehyde (4%) in acetic anhydride (containing a few crystals of sodium acetate), followed by heating at 130–150° for 1–2 min. (Gaffney *et al.* 1954).

Glycine. This was identified by the method of Consden, Gordon & Martin (1944). The solvent system used was phenol saturated with water (3% ammonia added in the tank) and the acid was detected with a ninhydrin spray reagent (0·1% in butan-1-ol).

Spectrophotometry

Ultraviolet-absorption spectra were measured with a model DK2 recording spectrophotometer (Beckman Instruments Inc., Calif., U.S.A.). The u.v.-absorption data for the derivatives and metabolites of BHT are set out in Tables 1 and 2 respectively. Infrared spectra of BHT and its metabolites were measured with a model IR2 Beckman recording spectrophotometer in KBr disks.

Metabolites of butylated hydroxytoluene

Urine from rabbits given BHT had pH 5·8–6·3 and reduced Fehling and Benedict reagents. It gave no colour reaction with aq. or ethanolic $FeCl_3$ soln. and did not react with 2:4-dinitrophenylhydrazine. By paper chromatography of the urine, compounds A, B, C and D were detected and some of their properties are shown in Table 2. Compound A was detected in ether extracts of urine as collected and B, C and D in ether extracts of urine at pH 2–3. BHT was not detected, and in some urines free glucuronic acid and glucuronolactone were present.

Isolation of 3:5-di-tert.-butyl-4-hydroxybenzoic acid. Urine (250 ml., pH 6·0) from a bulked supply collected over 7 days after a rabbit was given the first of five repeated daily doses of BHT (1 g.) was extracted with peroxide-free ether (4 × 100 ml.). The combined extracts were reduced *in vacuo* to yield a brownish-yellow crystalline deposit, m.p. 180–185° (0·32 g., 10·8% of the dose). This material was redissolved in ether (100 ml.) and then shaken out with $NaHCO_3$ soln. (aq. 5%); the acid was liberated from the bicarbonate solution and collected by extraction with ether (2 × 100 ml.). Repeated recrystallization from light petroleum (b.p. 60–80°) gave large colourless plates, m.p. 208–209° (0·2 g.), which was not depressed when admixed with authentic 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (Found: C, 72·4; H, 8·4%).

Preparation of the ether-soluble glucuronide fractions. Urine (200 ml.) from which the non-conjugated phenols had been removed (see above) was adjusted to pH 1·5–2·0 with dil. H_2SO_4 and extracted again with peroxide-free ether (3 × 200 ml.). The combined extracts were reduced *in vacuo* to a yellow–brown gum (1·0 g.), which gave a strongly positive test for both glucuronic acid and phenol (fraction 1). Paper chromatography showed the presence of glucuronides B and C as well as trace amounts of the glycine conjugate (compound D). The residual urine contained about 10% of the original glucuronide-reacting material. Continuous ether extraction removed this almost completely as well as the glycine conjugate. The solvent was removed *in vacuo* to yield a yellow–brown gum (60 mg.) (fraction 2). Paper chromatography showed the presence of compounds B, C and D.

Enzymic hydrolyses with β-glucuronidase. The β-glucuronidase powder was examined for hippuricase (aminoacylase 1) activity by the methods of Ellis & Walker (1942), hippuric acid being used as the substrate. At pH 3·1, 4·6 and 6·1 no free glycine was found after incubation for 2, 24 and 48 hr. with β-glucuronidase at 37°. The manufacturers stated that the enzyme preparation had no sulphatase activity on *p*-nitrophenyl sulphate.

(*a*) Rabbit urine. Urine (150 ml., pH 6·45), penicillin (10 000 units, Glaxo Crystapen) and β-glucuronidase powder (0·1 g., 5000 units) were incubated at 37° in a slow stream of N_2. After 6 hr. more β-glucuronidase powder (0·1 g.) was added and the incubation continued for a further 24 hr. with intermittent shaking. The crystalline ppt. was collected, dried and extracted with peroxide-free ether (2 × 100 ml.). Removal of the ether *in vacuo* gave an almost colourless crystalline product (0·22 g.), m.p. 170°, which was shown by paper chromatography with solvent system A (Table 1) to contain 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (R_F 0·45) and an unidentified substance (R_F 0·6). This was later shown to be identical with β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethanol), the aglycone of glucuronide C. The same compounds were identified in ether extracts of the residual urine.

(*b*) Ether-soluble glucuronide extract (fraction 1). Glucuronide gum (110 mg.) dissolved in 0·2M-sodium acetate buffer (25 ml., pH 4·5) was incubated with β-glucuronidase (13 mg., 660 units) at 37°. Samples were removed and the free glucuronic acid was determined by the cerimetric method of Levvy (1946). After 1 hr. 72% of the glucuronides were hydrolysed and 77% after 7 hr. In buffer of pH 5·5, 90% of the material was hydrolysed after 20 hr.

Glucuronide gum (0·5 g.) was incubated in acetate buffer (100 ml., pH 5·6) with β-glucuronidase powder (0·1 g., 5000 units) for 24 hr. at 37°. The crystals which separated (185 mg.) had m.p. 170–175°. Paper chromatography with solvent system A (Table 1) showed the same two compounds with R_F 0·45 and 0·6 as described in (*a*) above. Recrystallization from light petroleum (b.p. 60–80°) gave 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 210° unchanged by admixture with the authentic acid, and having the same u.v. spectrum in 95% (w/v) ethanol (Found: C, 72·5; H, 8·6%).

Table 1. *R_F values, colour reactions and absorption spectra of butylated hydroxytoluene and its derivatives*

Solvent mixtures (by vol., upper phases): A, *cyclo*hexane–methanol (2:1); B, *cyclo*hexane–benzene–methanol (90:10:1); C, ethyl acetate–pyridine–water (2:1:2). Colours of spots: B, blue; G, green; Gy, grey; M, magenta; O, orange; Y, yellow; d, deep; p, pale. Colour on keeping is given in parentheses.

	R_F value in solvent system			Colour with reagent			Light-absorption in 95% ethanol	
	A	B	C	Gibbs	Pauly	Molybdo-phosphate	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$
3:5-Di-*tert.*-butyl-4-hydroxytoluene (BHT)	0·85	0·9	0·9–1·0	pY*	None	pGB	208, 278, 284 ~ †	8030, 2200
3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid	0·45–0·5	0·8	0·9	M	YO	YG	214, 260	26560, 13145
3:5-Di-*tert.*-butyl-4-hydroxybenzaldehyde	0·65	0·8	—	pM	None	pYG	227·5, 290	18720, 16615
3:5-Di-*tert.*-butyl-4-hydroxybenzyl alcohol	0·4	0·8	0·9–1·0	M	Y	YG	249, 254·5, 260·5, 281 ~	—
3:5-Di-*tert.*-butyl-2:4-dihydroxytoluene	0·7	0·9	0·9–1·0	None (pY)	—	pGyB	224, 282·5, 286 ~	7180, 3260
Methyl 3:5-di-*tert.*-butyl-4-hydroxybenzoate	0·8	0·85	—	None (dB)	None	None	261	13440
β-(3-*tert.*-Butyl-2-hydroxy-5-methylphenyl)-$\beta\beta$-dimethylethanol	0·6	0·8–0·85	0·9–1·0	pY	None	pB	283	2790

* Colour is intensified on heating at 100° for 5 min.
† See also Tollenaar & Vos (1956); Phillips & Hinkel (1957).

Table 2. *R_F values, colour reactions and light-absorption of metabolites of butylated hydroxytoluene*

Solvent mixtures (by vol., upper phases): C, ethyl acetate–pyridine–water (2:1:2); D, lower phase of ethylene dichloride–*tert.*-butanol–acetic acid–water (75:25:30:70); E, butan-1-ol–water (1:1); F, butan-1-ol–2N-ammonia (1:1). Colours of spots: B, blue; M, magenta; Y, yellow; d, deep; p, pale. Colour on keeping is given in parentheses.

	R_F value in solvent system				Colour with reagent		Light-absorption in methanol
	C	D	E	F	Gibbs	Naphtha-resorcinol	$\lambda_{max.}$ (mμ)
Compound A (unconjugated phenol)	0·9	—	0·95–1·0	0·8	pM	None	212, 258
Compound B (ester glucuronide)	0·6	0·75–0·8	0·8	0·5–0·55	Y(M)	dB	210, 266·5
Compound C (ether glucuronide)	0·45–0·5	0·6 –0·65	0·7	0·2	B	dB	210, 277
Compound D* (glycine conjugate)	0·3 –0·35	0·25	0·5 –0·55	0·1	B(dB)	None	211, 261·5, 266 ~

* Orange–yellow with the Altman reagent.

Isolation of methyl [*β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*]*uronate*. From urine (500 ml.) of rabbits given BHT, the glucuronide gum from the basic lead acetate ppt. at pH 8 was prepared and methylated with diazomethane and then acetylated as described by Kamil, Smith & Williams (1951). Trituration of the dark gummy product with absolute ethanol produced large colourless plates (0·66 g.), m.p. 160–162°. Recrystallization from ethanol gave sheaths of long, fine colourless plates of *methyl* [*β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*]*uronate*, m.p. 167·5° (decomp.), $[\alpha]_D^{17·5}$ – 45·0° in $CHCl_3$ (*c*, 1·0); light-absorption max., 208 and 279 mμ (ε 10 490 and 2900 respectively) in 95% ethanol (Found: C, 60·7, 61·0; H, 7·2, 7·2; OMe, 5·7, 5·2; CH_3·CO, 24·2, 25·0; mol.wt., 541. $C_{28}H_{40}O_{11}$ requires C, 60·9; H, 7·3; OMe, 5·6; $3CH_3$·CO, 23·4%; mol.wt., 552).

Ether-soluble glucuronide gum (fraction 1) (0·5 g.), when methylated, acetylated and triturated as described above, yielded large colourless plates, m.p. 160° (0·2 g.). Recrystallization from *tert.*-butanol gave long flat colourless plates, or flat colourless prisms from methanol, m.p. 165·5° (decomp.) which was not depressed on admixture with the acetylated-methylated glucuronide from the lead acetate ppt. (Found: C, 60·6, 60·9; H, 7·0, 7·2; OMe, 6·3, 6·1%). This glucuronide was stable to boiling 0·5N-NaOH for 10 min.

Acid hydrolysis of methyl [*β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*]*uronate*. The crystalline glucuronide (280 mg.) in methanol (50 ml.) and 2N-HCl (25 ml.) was boiled under reflux for 3 hr. in a slow stream of N_2. On cooling, the mixture was extracted with light petroleum (b.p. 60–80°) (2 × 50 ml.). Removal of the solvent *in vacuo* gave long colourless crystalline plates (104 mg., 83%), m.p. 95–96°. Recrystallization from ethanol or sublimation *in vacuo* yielded colourless plates of *β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethanol*, m.p. 110° (Found: C, 76·5, 76·9; H, 10·6, 10·5; OMe, nil. $C_{15}H_{24}O_2$ requires C, 76·2; H, 10·25%).

Acid and enzymic hydrolysis of the crude ether glucuronide (*compound C*). Ether-soluble glucuronide gum (fraction 1) (200 mg.) was applied to Whatman no. 17 paper and run with solvent system C (Table 2). The band of compound C was cut out and eluted with methanol. Removal of the methanol *in vacuo* yielded a slightly yellowish gum which failed to crystallize from numerous solvents. After hydrolysis of the gum (about 20 mg.) by both methanolic HCl and *β*-glucuronidase (as described above), followed by paper chromatography of an ether extract, a compound with the same R_F in two solvent systems (A and B, Table 1) as *β*-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-*ββ*-dimethylethanol was identified.

Isolation of methyl (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*)*uronate*. The mother liquors remaining from the lead and ether-soluble glucuronide preparations after trituration with absolute ethanol and separation of the acetylated-methylated glucuronide of compound C, should contain the corresponding derivative of glucuronide B. Purifications and fractional separations in numerous solvents failed to effect crystallization of this compound. 3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid was isolated, however, after acid hydrolysis of this residual gum.

Ether-soluble glucuronide gum (fraction 1) (3·28 g.) from 670 ml. of fresh urine from rabbits given BHT (5 g.), was methylated with diazomethane and then acetylated in acetic anhydride (25 ml.) with perchloric acid (1 drop of aq. 60%) as described by Mead, Smith & Williams (1958). Trituration of the dried precipitated gum with absolute ethanol formed long colourless plates (0·84 g.) of methyl [*β*-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-*ββ*-dimethylethyl 2:3:4-tri-*O*-acetyl-*β*-D-glucopyranosid]uronate, m.p. 160° (decomp.); its identity was confirmed by a mixed-m.p. determination and u.v.-absorption spectrum. The residual gum (2·0 g.) was extracted with light petroleum (b.p. 60–80°) to remove any free 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid and after dissolving in methanol (40 ml.) was remethylated and reacetylated as described above. The dried gum, which contained microcrystals, m.p. 118–120° (micro-hot-stage), was dissolved in ethanol (10 ml.) and added to a column of acid-washed alumina. Three equal fractions were collected on elution with ethanol–acetic acid (1:1, v/v). The first fraction, after removal of the solvent *in vacuo*, yielded a slightly coloured amorphous mass (0·38 g.), which on trituration with absolute ethanol gave a mixture of colourless crystals and a glass-like gum. The crystals were separated and quickly washed with ethanol (42 mg., m.p. 130–131°). Recrystallization from ethanol yielded fine colourless needle-like plates of *methyl* (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*)*uronate*, m.p. 136–137° (decomp.), $[\alpha]_D^{20}$ – 35·0° in methanol (*c*, 1·0); light-absorption max., 217 and 267·5 mμ (ε 24 200 and 17 970 respectively) in 95% ethanol [Found: C, 59·8; H, 6·9; OMe, 5·8; CH_3·CO, 22·8 (corrected value: a blank determination with 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid gave an acetyl value of 6·4). $C_{28}H_{38}O_{12}$ requires C, 59·4; H, 6·8; OMe, 5·5; $3CH_3$·CO, 22·8%]. A mixed-m.p. determination with the acetylated-methylated glucuronide from the urine of rabbits given 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid was not depressed, but a mixed m.p. with the acetylated-methylated glucuronide of compound C (m.p. 167°) was lowered to 121–125°.

Acid hydrolysis of methyl (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*)*uronate*. The crystalline glucuronide (100 mg.) in methanol (25 ml.) and 2N-HCl (25 ml.) were boiled under reflux for 4 hr. in a slow stream of N_2. On cooling, water (25 ml.) was added and the mixture extracted with peroxide-free ether (3 × 50 ml.). Removal of the ether *in vacuo* after drying over anhydrous $MgSO_4$ gave yellowish crystals (38 mg., 86·4%), m.p. 185–187°. Paper chromatography with solvent system A (Table 1) showed the presence of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid and a trace of methyl 3:5-di-*tert.*-butyl-4-hydroxybenzoate. Vacuum sublimation of the crystals gave pure 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 206–207° which was not depressed on admixture with the authentic acid.

Acid and enzymic hydrolysis of the crude ester glucuronide (*compound B*). Ether-soluble glucuronide gum (fraction 1) (200 mg.) was applied to Whatman no. 17 paper and run with solvent system C (Table 2). The band of compound B was separated and eluted with methanol. Removal of the methanol *in vacuo* yielded a slightly yellow gum which failed to crystallize from numerous solvents, even after rechromatography. After hydrolysis of the gum (about 20 mg.) by both methanolic HCl and *β*-glucuronidase, followed by paper chromatography of an ether extract and

of the residual mother-liquor concentrates, the only compound identified was 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid.

Acid hydrolysis of the glycine conjugate (compound D). Ether-soluble gum (fraction 2) (40 mg.) was applied to paper and developed with solvent system C (Table 2). The band of the glycine conjugate was cut out and eluted with water (10 ml.). After hydrolysis with HCl, both glycine and 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid were identified by chromatographic analysis.

Metabolites of 3:5-*di*-tert.-*butyl*-4-*hydroxybenzoic acid*

Isolation of unconjugated 3:5-*di*-tert.-*butyl*-4-*hydroxybenzoic acid.* Urine (400 ml., pH 5·85) from a rabbit given 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (1·95 g.) in three consecutive daily doses, was extracted by shaking with peroxide-free ether (2 × 400 ml.). The extract was dried over anhydrous $MgSO_4$ and the solvent taken off *in vacuo* to yield a slightly yellow crystalline product. Vacuum sublimation (100–110°/0·05 mm. Hg) gave colourless crystalline plates (38 mg., 2·0 % of the dose), m.p. 210° which was undepressed on admixture with authentic 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid. No other compound was detected in the ether extract by paper chromatography in solvent system A (Table 1).

Isolation of methyl (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-β-D-*glucopyranosid*)*uronate.* Urine (400 ml.) after removal of the non-conjugated phenols (see above) was adjusted to pH 2·2 with dil. HCl and extracted by shaking with peroxide-free ether (3 × 400 ml.). The extract was dried over anhydrous $MgSO_4$ and the solvent removed *in vacuo* to yield a white friable powder (2·75 g., 93·9 %). Chromatographic examination of this material with solvent systems C, E and F (Table 2) gave two spots with R_F values and colour reactions identical with those of compounds B and D (Table 2). No other glucuronide-reacting compounds nor any other metabolites were detected in the residual urine, which gave negative Fehling and Benedict tests. The ether-soluble powder (1·6 g.) was methylated with diazomethane and acetylated with perchloric acid as described above. The precipitated gum was washed, dried and then triturated with absolute ethanol. The completely crystalline product (1·8 g.), m.p. 127–128°, was repeatedly recrystallized from ethanol to yield long colourless needles or prisms of methyl (3:5-di-*tert.*-butyl-4-hydroxybenzoyl-2:3:4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate, m.p. 137·5–138° (decomp.) undepressed on admixture with the methylated–acetylated glucuronide of compound B from BHT urine, $[\alpha]_D^{20}$ −35·0° in methanol (*c*, 1·0) (Found: C, 59·4, 59·9; H, 6·8, 7·0; OMe, 6·3 %).

Acid hydrolysis of the glycine conjugate. The ether-soluble powder (150 mg.) was applied to paper and run with solvent system C (Table 2). The band of the glycine conjugate was cut out and eluted with water (15 ml.). After hydrolysis, both glycine and 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid were identified.

RESULTS

Excretion of normal metabolites. The mean daily excretions (± S.D.), by undosed rabbits were: free phenols, 0·544 ± 0·135; total phenols, 0·667 ± 0·177; total glucuronides, 0·366 ± 0·184; ester glucuronides, 0·230 ± 0·058; ethereal sulphates, 0·164 ± 0·080; glycine conjugates, 0·323 ± 0·103 m-mole.

Metabolites of butylated hydroxytoluene

The results of the analytical determinations of metabolites excreted in rabbit urine after the administration of single and repeated daily doses of butylated hydroxytoluene are set out in Table 3. Excretion of all the metabolites is complete only after 3–4 days after dosing of the animals. About 54 % of the administered dose is accounted for, and the recovery of metabolites after four and five repeated doses is not very different from that of single doses of butylated hydroxytoluene.

All the metabolites of butylated hydroxytoluene as indicated by the initial analyses, with the exception of the ethereal sulphates, were satisfactorily accounted for by the paper-chromatographic examination of urine. Methods for the location and identification on paper chromatograms of the ethereal sulphates were not successful.

Non-conjugated phenols. When urines were ether-extracted at pH 5·5–6·5, only one compound was present in the extract. This was shown to be identical with 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid. No unequivocal evidence for butylated hydroxytoluene by chromatographic examinations of solvent extracts or steam-distillates of urine was found and if any is excreted the amount is likely to be very small.

Glucuronide conjugates. The excretion of glucuronides accounts for almost 36 % of doses of butylated hydroxytoluene (Table 3) and 16 % could be attributed to an ester-type glucuronide (compound B). Paper-chromatographic analysis also confirmed the excretion of two different glucuronides (Table 2). These two metabolites were isolated from urine as the crystalline methylated–acetylated derivatives. The ester glucuronide (compound B) was shown to be identical by m.p., mixed m.p., chromatographic behaviour and u.v. spectrum with the fully acetylated-methylated glucuronide isolated from the urine of rabbits given 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (see below). The compound is thus formulated as 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid. Elementary analysis of the acetylated–methylated ether glucuronide (compound C) and its crystalline aglycone indicated oxidation of the butylated hydroxytoluene molecule with one atom only of oxygen. The i.-r. spectrum of both the aglycone and glucuronide showed the presence of strong bands at 2·90 μ (the sterically hindered hydroxyl group), at 9·45 μ (a primary alcohol side chain) and at 12·90 μ (a phenolic *C*-methyl group). The spectra support the contention that oxidation of a *tert.*-butyl group to a (1-hydroxy-2:2-dimethyl)ethyl group has taken place. On this evidence the aglycone is

Table 3. *Metabolites excreted in rabbit urine after the administration of single or repeated daily doses of butylated hydroxytoluene*

Results are expressed as a percentage of the dose (single animals). Free phenols: ester glucuronide + non-conjugated phenols. Values obtained by difference are in parentheses.

No. of doses	Daily dose (m-moles/kg.)	Non-conjugated phenols	Free phenols	Total phenols	Total glucuronides	Ester glucuronide	Ether glucuronide	Ethereal sulphate	Glycine conjugate
1	2·28	—	26·2	31·7	35·5	—	—	5·3	—
1	2·43	—	22·4	24·4	38·7	—	—	9·0	—
1	3·64	4·1	19·9	22·0	35·4	18·4	(17·0)	9·9	—
4	1·82	9·6*	—	—	—	—	—	—	—
4	3·65	—	—	—	33·2	14·9	(18·3)	—	—
5	3·63	—	21·2	—	36·1	16·0	(20·1)	7·6	—
5	3·64	8·0	—	—	—	—	—	10·3	1·8
5	3·64	—	30·9	30·7	36·6	14·5	(22·1)	6·0	—
	Mean	8·4	24·1	27·2	35·9	16·0	(19·4)	8·0	1·8

* Mean of four separate experiments where only the non-conjugated phenols were determined.

identified as β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethanol and the glucuronide is formulated as β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethyl β-D-glucosiduronic acid. The aglycone has not yet been synthesized.

Glycine conjugate. This metabolite (compound D) was shown to be chromatographically identical with the glycine conjugate that is present in urine after the administration of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid to a rabbit. Hydrolysis of the conjugate eluted from paper chromatograms confirmed that its structure is 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

Ethereal sulphates. About 8 % (± 1·9) of doses of butylated hydroxytoluene were excreted as ethereal sulphates (see Table 3).

Mercapturic acids. No consistent indication of the excretion of butylated hydroxytoluene in the form of mercapturic acid conjugates was found.

Metabolites of 3:5-*di*-tert.-*butyl*-4-*hydroxybenzoic acid*

After the oral administration of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid in three consecutive daily doses to a rabbit, more than 80 % was accounted for as ester-type glucuronide and about 9 % as a glycine conjugate. Excretion of metabolites was normal on the fifth day after dosing commenced. The recovery of the metabolites is set out in Table 4. The glucuronide was 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid and the glycine conjugate was 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

Table 4. *Metabolites excreted in rabbit urine after the administration of* 3:5-*di*-tert.-*butyl*-4-*hydroxybenzoic acid daily over three consecutive days*

Results are expressed as a percentage of the dose (single animals).

	Total dose (m-moles/kg.)	
	2·25	4·70
Non-conjugated phenols	5·2	2·0
Free phenols (non-conjugated phenol + ester glucuronide)	90·5	—
Total phenols	96·2	—
Total glucuronides	84·0	87·5
Ester-type glucuronide	83·5	—
Glycine conjugate	8·8	—

DISCUSSION

The usual methods employed for the estimation of phenols in biological materials (Bray & Thorpe, 1954) cannot be used for butylated hydroxytoluene because of the sterically hindered hydroxyl group. Although the compound reacts with ferric chloride-

2:2′-dipyridyl (Anglin, Mahon & Chapman, 1956; Eastman Chemical Products Inc., 1957), in our hands the method was not sufficiently accurate nor applicable to the routine analysis of butylated hydroxytoluene in urine samples. No free butylated hydroxytoluene was found by the ferric chloride–2:2′-dipyridyl method, nor was it seen in any of the paper chromatograms. Similarly, there was no evidence of conjugates hydrolysable to butylated hydroxytoluene itself. Furthermore, no evidence was found for the conjugation of the metabolites of butylated hydroxytoluene in the normal manner through the hindered hydroxyl group. Confirmation of a free unconjugated hydroxyl group was obtained from measurements of the infrared spectra of these metabolites (cf. Bohn & Campbell, 1957).

From the metabolites isolated it is clear that butylated hydroxytoluene is oxidized either by conversion of the methyl group in position 1 into a carboxyl group or by ω-oxidation of one of the *tert.*-butyl groups to the primary alcohol stage, and that the conjugates are formed through one of these two new groups. As the phenolic hydroxyl of butylated hydroxytoluene is sterically hindered, the pathway by ω-oxidation is probably the one followed for ethereal sulphate synthesis. The conjugation of certain types of primary alcohols with sulphuric acid is known to occur (Williams, 1959). Both types of oxidation have been reported previously: about 10% of an oral dose of *p*-cresol is excreted as free and conjugated *p*-hydroxybenzoic acid (Bray, Thorpe & White, 1950) and up to 90% of a dose of *tert.*-butylbenzene is oxidized by rabbits to 2:2-dimethyl-2-phenylethanol and excreted as a glucuronide (Robinson & Williams, 1955). As far as we are aware, this is the first example of a single compound following both oxidative routes and involving three different conjugating agents.

The study has shown that rabbits can cope with relatively large single doses of butylated hydroxytoluene (Table 3) and up to three daily doses of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (Table 4). That four and five consecutive daily doses of butylated hydroxytoluene have cumulative and lethal effects is, however, not very reassuring. The metabolites described account for 54·1% (±7·2%) of the dose. The fate of the remainder is unknown.

SUMMARY

1. The metabolism of the antioxidant 3:5-di-*tert.*-butyl-4-hydroxytoluene (BHT) and its oxidation product, 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, has been studied in the rabbit.

2. Rabbits excreted BHT mainly as glucuronides, some 16·0% as an ester glucuronide and 19·4% as an ether glucuronide. Unconjugated phenol (8·4%), ethereal sulphate (8·0%) and a glycine conjugate (1·8%) were also excreted. The distribution and recovery of metabolites when four and five daily doses were given was not significantly different from that of single doses of BHT.

3. After three consecutive daily doses of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, a rabbit excreted 84·0% of the total dose as ester glucuronide, 8·8% as a glycine conjugate and 5·2% was not conjugated.

4. The ester glucuronide, isolated from the urine after both BHT and the acid as the triacetyl methyl ester, is 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid.

5. The ether glucuronide, isolated from BHT urine as the triacetyl methyl ester, is β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-$\beta\beta$-dimethylethyl β-D-glucosiduronic acid.

6. The glycine conjugate of both BHT and the acid is 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

The author is greatly indebted to Professor F. A. Denz, Pathology Department, for many valuable discussions and encouragement during the course of this investigation, and to Professor L. H. Briggs, Chemistry Department, University of Auckland, for the measurements of the infrared spectra.

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Biochem. J. (1961) **78**, 766

Metabolic Effects of α-Oxobutyric Acid: Effect on Respiration of Rat-Liver Homogenates

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(*Received* 3 *May* 1960)

The observation that the plasma level of α-amino-*n*-butyric acid is elevated in several pathological states (Iyer, 1956) stimulated interest in the metabolism of the amino acid. This amino acid has been shown to participate in transamination reactions (Iyer & Sukumaran, 1959). It was felt that a study of the metabolism of the corresponding keto acid, α-oxobutyric acid, would help to explain some of the observations.

α-Oxobutyric acid arises chiefly from methionine (Greenberg, 1954) and, probably, also from threonine (Lien & Greenberg, 1952). Long (1942, 1943) has demonstrated that pyruvic-oxidase preparations from brain oxidize α-oxobutyric acid at the same rate as pyruvate. Kinnory, Takeda & Greenberg (1955) have shown that the first step in the catabolism of α-oxobutyric acid is its oxidative decarboxylation to propionic acid. One would therefore expect α-oxobutyric acid to cause an increase in oxygen uptake of liver tissue, which would be a measure of the keto acid catabolized. On this premise we made a manometric study of the metabolism of α-oxobutyric acid. Contrary to expectation, inhibition rather than stimulation of oxygen consumption occurred on the addition of α-oxobutyric acid to rat-liver homogenate.

Further, α-oxobutyric acid causes an appreciable depression in oxygen uptake of rat liver when α-oxoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, glutamate or aspartate is present as a substrate. α-Oxobutyric acid has been shown to inhibit the formation of citrate from added oxaloacetate and also the utilization of α-oxoglutarate.

MATERIALS AND METHODS

Adult rats were killed by a blow on the head and the liver was dissected out. The liver was rinsed free of blood in ice-cold Krebs–Ringer phosphate solution (Krebs & Eggleston, 1940) and then homogenized in ice-cold Krebs–Ringer phosphate solution containing cytochrome *c* and diphosphopyridine nucleotide (DPN), or in 1·15% (w/v) potassium chloride solution, in a Potter–Elvehjem grinder. Preliminary observations on the depression of endogenous respiration were made with a 30% homogenate in potassium chloride medium. A 10% homogenate in the other medium was subsequently used for studying the effect of α-oxobutyric acid on substrate-stimulated respiration, since such a preparation had the advantage of a relatively low endogeneous respiration and a fairly high substrate-stimulated respiration.

Solutions of the potassium salts of citric acid, α-oxoglutaric acid, succinic acid, fumaric acid, L-malic acid, L-glutamic acid, L-aspartic acid, pyruvic acid and α-oxobutyric acid (L. Light and Co. Ltd.) were prepared by neutralizing their aqueous solutions and adjusting the pH to 7·3 with N-potassium hydroxide in the cold. The final concentrations used are given in the tables.

The oxygen consumption was measured by conventional Warburg manometry at 38° after allowing 3 min. for

equilibration. The gas phase was oxygen. Respiration was linear for the first 45 min. of incubation.

α-Oxoglutarate was determined by the method of McArdle (1957). Immediately after the measurement of oxygen consumption, 1 ml. of the reaction mixture was added to 20 ml. of 2·5% metaphosphoric acid, the volume was made to 25 ml., and α-oxoglutarate in 20 ml. of the protein-free filtrate was determined as its dinitrophenylhydrazone. The dinitrophenylhydrazone of α-oxobutyric acid resolves itself into two bands (R_F 0·86 and 0·64), which are well separated from that of α-oxoglutaric acid (R_F 0·36). The solvent run was 15 cm.

Citric acid was estimated by the method of Ettinger, Goldbaum & Smith (1952) with light petroleum (b.p. 40–60°) instead of heptane for the extraction of pentabromoacetone.

RESULTS

Respiration of liver homogenates. α-Oxobutyric acid depressed the endogenous oxygen consumption of liver homogenates (Table 1). Oxygen consumption stimulated by the addition of citrate, α-oxoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, glutamate or aspartate was also depressed (Table 2). With the Krebs-cycle intermediates, the percentage inhibition varied in a somewhat regular fashion with the substrate used. The maximum inhibition (88%) was observed with oxaloacetate as the substrate and the minimum (7%) with citrate. The inhibitory effect of α-oxo-

Table 1. *Effect of α-oxobutyrate on oxygen uptake by liver homogenates*

Volume of 30% homogenate in each flask, 1 ml.; final volume of the reaction mixture was made to 2 ml. with water; final concentration of additions are given in parentheses. Duration 30 min. Temperature of incubation 38°. Gas phase O_2.

Additions	Consumption of oxygen (μl./mg. of dry tissue/hr.) Range	Mean (6 expts.)	Inhibition (%)
None	4·1– 6·5	5·2	—
Oxobutyrate (25 mM)	1·9– 4·0	2·9	44
Oxaloacetate (12·5 mM)	4·9–12·2	7·7	—
Oxaloacetate (12·5 mM) + oxobutyrate (25 mM)	1·0– 2·6	1·6	79

Table 2. *Effect of α-oxobutyrate on oxygen uptake by liver homogenates in the presence of added substrates*

Each flask contained 1 ml. of 10% homogenate containing cytochrome *c* and DPN at final concentrations 0·05 mM and 1 mM respectively; water was added to make the final volume 2 ml.; final concentrations of added substrate and of oxobutyrate, 12·5 mM; duration 60 min. Temperature of incubation 38°. Gas phase O_2.

Additions	Uptake of oxygen (μl./mg. of dry tissue/hr.) Range	Mean (6 expts.)	Inhibition (%)
None	3·0– 6·6	4·5	—
Oxobutyrate	1·6– 3·2	2·4	47
Citrate	9·5–18·1	14·5	—
Citrate + oxobutyrate	9·0–15·0	13·5	7
α-Oxoglutarate	10·1–23·1	17·0	—
α-Oxoglutarate + oxobutyrate	9·5–17·7	13·4	21
Succinate	10·7–20·1	17·7	—
Succinate + oxobutyrate	5·3–10·2	8·3	53
Fumarate	9·9–18·6	15·0	—
Fumarate + oxobutyrate	2·8– 4·1	3·5	77
Malate	10·0–19·2	15·3	—
Malate + oxobutyrate	2·9– 4·0	3·5	77
Oxaloacetate	9·8–22·1	15·8	—
Oxaloacetate + oxobutyrate	1·5– 2·4	1·9	88
Glutamate	13·6–17·7	15·2	—
Glutamate + oxobutyrate	10·4–14·6	12·8	16
Aspartate	11·9–14·4	13·2	—
Aspartate + oxobutyrate	1·6– 2·8	2·2	83
Pyruvate	13·8–19·5	16·0	—
Pyruvate + oxobutyrate	3·6– 4·6	4·1	74

Table 3. *Effect of α-oxobutyrate on (a) citrate synthesis and disappearance and (b) α-oxoglutarate formation and disappearance*

Experimental conditions were as given in Table 2. Final concentration of oxobutyrate, 12·5 mM; concentrations of substrate are given in parentheses.

	$10^2 \times$ Changes (μmoles/mg. of dry tissue/hr.)			
	(*a*) Citrate		(*b*) α-Oxoglutarate	
Additions	Range	Mean	Range	Mean
Oxaloacetate (12·5 mM)	4·15–13·0	+ 7·32	—	—
Oxaloacetate (12·5 mM) + oxobutyrate	0·85– 3·3	+ 2·0	—	—
Oxaloacetate (25 mM)	12·10–24·0	+18·10	—	—
Oxaloacetate (25 mM) + oxobutyrate	2·76– 4·50	+ 3·85	—	—
Oxaloacetate (50 mM)	30·93–44·10	+36·07	—	—
Oxaloacetate (50 mM) + oxobutyrate	7·98–14·14	+10·90	—	—
Citrate (12·5 mM)	41·75–48·75	−45·0	6·1– 8·36	+ 7·05
Citrate (12·5 mM) + oxobutyrate	50·0 –62·0	−56·75	13·5–31·4	+25·0
α-Oxoglutarate (12·5 mM)	—	—	62·0–76·50	−67·56
α-Oxoglutarate (12·5 mM) + oxobutyrate	—	—	40·0–67·50	−54·60

butyrate on the oxygen consumption appeared to be enhanced by added oxaloacetate, for the oxygen consumption in the presence of both oxobutyrate and oxaloacetate was smaller than with oxobutyrate alone. This effect was very marked with the 30% homogenate (Table 1). α-Oxobutyrate depressed the oxygen uptake of liver homogenates stimulated by glutamate and α-oxoglutarate to nearly the same extent. A similar observation was made with aspartate and oxaloacetate.

Citrate synthesis. The amount of citrate formed from oxaloacetate, under the conditions of these experiments, increased in proportion to the concentration of added oxaloacetate (Table 3). α-Oxobutyrate inhibited citrate formation and at a constant concentration of the inhibitor (12·5 mM) in the medium, the citrate synthesized was directly related to the concentration of oxaloacetate.

Utilization of citrate and α-oxoglutarate. Aerobic incubation of liver homogenates with citrate produced α-oxoglutarate. When the incubation was carried out in the presence of α-oxobutyrate (12·5 mM), larger amounts of α-oxoglutarate were found (Table 3). α-Oxobutyrate (12·5 mM) inhibited the removal of added α-oxoglutarate (Table 3), and also enhanced the disappearance of citrate.

DISCUSSION

The principal finding in this investigation is that α-oxobutyrate depresses the endogenous respiration of rat-liver homogenates. It also inhibits respiration stimulated by several added substrates. The question arises whether α-oxobutyrate interferes with oxidation at many sites or at one single site. That oxobutyrate has been shown here to inhibit respiration stimulated by various substrates strongly suggests that it acts at a number of different sites. However, the possibility of a single, or major, site of action must also be considered. Bearing on this possibility are the two observations that: (1) it inhibits citrate formation from added oxaloacetate, and (2) among the Krebs–cycle intermediates there is a progressive increase in the inhibitory action of oxobutyrate on the oxidation, in the order citrate, α-oxoglutarate, succinate, fumarate, malate, oxaloacetate; the inhibition was greatest with oxaloacetate.

Oxaloacetate has been shown to have the capacity to inhibit a number of reactions within the citric acid cycle. Potter (1946) demonstrated its ability to inhibit malic dehydrogenase and its inhibitory action on succinic dehydrogenase is well established (Das, 1937; Pardee & Potter, 1948; Brummond & Burris, 1953; Tyler, 1955*a*, *b*; Wolff & Ball, 1957; Avron & Biale, 1957; Corwin & Schwarz, 1959). Tyler (1960) has shown that it also inhibits the removal of α-oxoglutarate. Such data suggest that α-oxobutyrate might exert its effect by inhibiting the synthesis of citrate and thereby the removal of oxaloacetate rather than by inhibiting each individual step in the citric acid cycle. The accumulation of oxaloacetate, in turn, would inhibit oxidative processes in the cycle. Such a rate-regulating function for oxaloacetate has been recently proposed by Tyler (1957, 1960). He suggested that substances affecting succinate oxidation and so oxidative activity through the cycle do so indirectly by affecting oxaloacetate metabolism. These observations here support this possibility, particularly since it is now well established that very small changes in oxaloacetate concentration have profound effects on succinate oxidation (Pardee & Potter, 1948; Tyler, 1955*b*). On this basis one would expect a more marked depression of oxygen uptake by a combination of oxaloacetate and α-oxobutyrate than by the latter alone; this is borne out by the data in Tables 1 and 2.

Interference with the removal of oxaloacetate through citrate formation would naturally impair the catalytic oxidation of pyruvate. The inhibition of oxygen uptake stimulated by aspartate may well be another consequence of the same effect. A similar explanation can be offered for the inhibitory effect on glutamate oxidation, since oxobutyrate inhibits the removal of α-oxoglutarate as well.

α-Oxobutyrate, which inhibits the utilization of α-oxoglutarate, presumably enhances its formation from added citrate. Montgomery & Webb (1956) have reported a similar effect with another inhibitor of oxoglutarate oxidation, namely parapyruvate, in the presence of which the unaffected sections of the Krebs cycle proceed at a more rapid pace.

SUMMARY

1. α-Oxobutyric acid depresses the endogenous oxygen consumption of rat-liver homogenates.

2. Respiration stimulated by citrate, α-oxoglutarate, succinate, fumarate, malate, oxaloacetate, glutamate, aspartate or pyruvate is also depressed, the extent of this varying with the substrate used. It is maximal with oxaloacetate and minimal with citrate.

3. Inhibition has been observed at two sites in the cycle: (i) between oxaloacetate and citrate and (ii) between α-oxoglutarate and succinate. α-Oxobutyrate decreases citrate formation from added oxaloacetate and also removal of added α-oxoglutarate.

4. The implications of these findings are discussed.

The authors appreciate the kindness of Professor D. B. Tyler in making available a typescript of his paper whilst it was in the press. They also acknowledge the encouragement of Dr M. Thangavelu, the Principal of this College.

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Biochem. J. (1961) **78**, 769

The Determination of Human-Serum-Cholinesterase Activity with *o*-Nitrophenyl Butyrate

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(*Received 5 May* 1960)

The increasing use of organophosphorus compounds in agriculture, forestry and medicine has placed great emphasis on the need for a rapid and reasonably accurate method for determining human-serum-cholinesterase activity. The present paper describes such a method based on the use of *o*-nitrophenyl butyrate as substrate. Cholinesterase liberates *o*-nitrophenol under easily reproduced, relatively non-critical conditions. The *o*-nitrophenol can be measured spectrophotometrically, and only a small volume (0·004 ml.) of serum is needed for each determination.

The effect of the orientation of the nitro group of nitrophenyl carboxylic esters on the cholinesterase activity was studied. Previously it had been shown that the A-type esterases in sheep serum hydro-

lysed *ortho*-orientated esters at a slower rate than the *meta* and *para* isomers (Main, 1960). The A-esterases in human serum showed a similar specificity. In the present work it was observed that cholinesterase hydrolysed *ortho*-nitro esters more rapidly than the corresponding *para* esters. Aldridge (1953) has shown that human serum contains only A-type esterases and cholinesterases. In addition he has shown that A-type esterases hydrolyse acetate esters more readily than butyrate esters, whereas Adams & Whittaker (1949) have shown that human-serum cholinesterase hydrolyses butyrates in preference to acetates. The application of these specificity studies has resulted in the synthesis of a compound, *o*-nitrophenyl butyrate, which is highly specific for cholinesterase in human serum.

EXPERIMENTAL

Materials

Preparation of nitrophenyl esters. *p*- and *m*-Nitrophenyl esters were prepared according to the Aldridge (1953) modification of the method of Huggins & Lapides (1947). *o*-Nitrophenyl acetate was a commercial preparation.

Preparation of o-nitrophenyl butyrate. This could not be prepared by the method of Huggins & Lapides (1947). Accordingly, the procedure given below was developed.

The sodium salt of *o*-nitrophenol was prepared by adding twice the stoicheiometric amount of a 75% (w/v) NaOH solution to a 25% (w/v) solution of *o*-nitrophenol in methanol at room temperature. The sodium salt was precipitated with about 90% yield. It was filtered on a Büchner funnel and washed three times with a small volume of methanol, once with ether, and dried in the air.

The sodium salt (16·2 g., 0·1 mole) was mixed with 30 ml. of wet benzene in a round-bottom flask and shaken to form a slurry. Butyryl chloride (14·5 ml., 0·14 mole) was added slowly with shaking at room temperature. As the reaction proceeded, the colour changed from red to yellow and NaCl was precipitated. When all the butyryl chloride had been added, the flask was placed under a reflux condenser, and the mixture was refluxed gently for 1 hr. After cooling, 3 vol. (approx. 150 ml.) of ether were added, and the solution was transferred to a 500 ml. separating funnel.

The organic phase was washed twice with water, three times with 1% aq. $NaHCO_3$, once with 1% citric acid and twice with water. The volume of each wash was about 200 ml. The organic phase was then transferred to a 250 ml. conical flask, and after drying over Na_2SO_4 (about 20 g.), it was filtered into a distillation flask, and the benzene ether components were distilled off under a partial vacuum, (approx. 100 mm. Hg). The yield from the sodium salt was about 85%.

The *o*-nitrophenyl butyrate was contaminated with *o*-nitrophenol. This was removed by recrystallizing the *o*-nitrophenyl butyrate three or four times from a 25% (v/v) solution in diethyl ether at the temperature of a solid CO_2–acetone bath. The freezing point of *o*-nitrophenyl butyrate was too low to permit crystallization at higher temperatures. Crystallization had to be initiated by seeding with crystals of *o*-nitrophenyl butyrate. These were obtained from a previously frozen solution in methanol. Although crystallization from methanol was much more rapid than from ether, the crystals obtained were heavily contaminated.

The freezing point of the purified *o*-nitrophenyl butyrate appeared to be about −20°; but unless the liquid was seeded, it remained liquid at this temperature. The ether was decanted from the crystal crops, and traces were removed from the last crop by evaporation under vacuum (60 mm. Hg) at 50°. The *o*-nitrophenyl butyrate decomposed before it would distil at 760 mm. Hg, and even under low pressure (2–3 mm. Hg) it tended to decompose and give *o*-nitrophenol in the distillate. The *o*-nitrophenyl butyrate prepared as described was 99·5–99·7% pure and contained 0·4–0·2% of *o*-nitrophenol. This amount of *o*-nitrophenol gave a negligible reagent blank at the *o*-nitrophenyl butyrate substrate concentrations normally used.

The density of *o*-nitrophenyl butyrate at 25° was 1·147.

Phosphate buffer. Most reactions were carried out at pH 7·6 in 50 mM-phosphate buffer. For convenience a 500 mM-solution was prepared by adding 9 g. of NaH_2PO_4,H_2O and 160 g. of $Na_2HPO_4,12H_2O$ and diluting to 1 l. This solution was diluted tenfold when required.

Diisopropyl phosphorofluoridate. Di*iso*propyl phosphorofluoridate (DFP) (0·10 ml.) was diluted to 25 ml. with propan-2-ol. The concn. of DFP was about 25 mM.

Buffered substrates. According to Aldridge (1953), the solubility of *p*-nitrophenyl carboxylic esters is about one-tenth of the values given by Huggins & Lapides (1947). The solubility of *p*-nitrophenyl acetate would then be approx. 3 mM, and of the *p*-nitrophenyl butyrate, 1 mM at 25°. Solution of these esters required special attention. It has been observed that *o*- and *p*-nitrophenyl acetates in concentrations as high as 5 mM could be prepared if a suitable procedure such as the one below was followed.

Stock solutions of the esters (usually 0·5 M) in methanol or other convenient alcohol were prepared. These solutions were stable for weeks if the alcohol used was first adjusted to about pH 5 with acetic acid.

Appropriate volumes of stock solution were blown into the aqueous medium with the tip of the pipette below the liquid surface as recommended by Huggins & Lapides (1947). Solution was more effective if the stock was blown into salt-free water. When concentrations of about 5 mM were desired, preheating the water to 32° was necessary. The dissolved substrate solution was then cooled and a suitable volume of concentrated phosphate buffer and acacia was added.

Human serum. Samples of human blood were withdrawn from volunteers by hypodermic syringe and defibrinated by stirring with a glass rod to which the clot adhered. The blood was collected in polyethylene test tubes and immediately centrifuged to separate the serum. The serum was stored frozen.

For routine testing, samples of blood were taken by pricking the finger and collecting in a capillary tube, which had previously been soaked in 0·1% heparin solution and dried. The plasma was separated by sealing one end of the capillary and centrifuging. Approx. 0·05 ml. of plasma was obtained in this way.

Determination of enzyme activity

Spectrophotometric method. The rate of *o*-nitrophenyl butyrate hydrolysis was determined by measuring the concentration of *o*-nitrophenol liberated in a given time either with a Hilger Uvispek or with a Beckman DK-2 ratio

recording spectrophotometer, equipped with time drive and temperature-controlled cell compartment.

In the presence of *o*-nitrophenyl butyrate, *o*-nitrophenol can be measured at two absorption peaks as shown in Fig. 1. One peak at 348 mμ is the absorption of the un-ionized form, and the other at 414 mμ is the absorption of the ionized oxide form. Since the pK of *o*-nitrophenol is pH 7·1, the ionized form predominates above and the un-ionized below this pH. The isosbestic point, at which absorption is independent of pH, was 371 mμ. When the Hilger Uvispek was used, the *o*-nitrophenol concentration was a linear function of absorption up to an extinction of 1·5.

The absorption spectra of *o*-nitrophenyl butyrate was independent of the pH. As shown in Fig. 1, it absorbed at and below the wavelength of the *o*-nitrophenol isosbestic point.

In the present work, it proved more convenient to measure *o*-nitrophenol concentrations at 414 mμ and pH 7·6 where the sensitivity is 1·5 as high as at the isosbestic point and where the *o*-nitrophenyl butyrate absorption was negligible. The sensitivity could be further increased by bringing the pH above 9, but the spontaneous hydrolysis of *o*-nitrophenyl butyrate at such pH values creates large reagent blanks. The *o*-nitrophenol absorption was calibrated for each new preparation of buffer, in order to correct for small pH variations. Colorimetric determinations were all carried out at 25° in a constant-temperature bath. Careful temperature control, within $\pm 0{\cdot}1°$, was necessary to achieve the highest accuracy.

The cholinesterase-catalysed hydrolysis rates of *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate were also determined. The liberated *p*-nitrophenol was measured at its isosbestic point (345 mμ). The absorption spectra of *p*-nitrophenol and of its acetate are given by Bergmann, Rimon & Segal (1958) who reported that the *p*-nitrophenol isosbestic point was 348 mμ.

A-Esterase activities were measured with serum dilutions which had previously been incubated with 0·1 mM-DFP. The reagent blank contained an equivalent amount of serum dilution which had previously been acidified and boiled to destroy all enzymic activity. The A-esterase activity was the difference in absorption between the reaction medium containing the DFP serum dilution and the boiled enzyme blank.

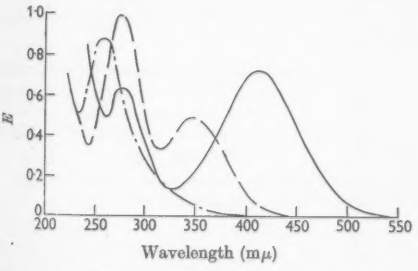

Fig. 1. Absorption spectra of *o*-nitrophenol and of *o*-nitrophenyl butyrate. The *o*-nitrophenol and *o*-nitrophenyl butyrate concentrations were 0·15 mM. The *o*-nitrophenol (un-ionized) spectrum at pH 4·5 (– –) and the *o*-nitrophenyl butyrate spectrum (– – –) were determined in 20 mM-acetate buffer. The *o*-nitrophenol (ionized) spectrum at pH 9 (—) was determined in 20 mM-borate buffer. The spectra were determined at 25° with the Beckman DK-2 ratio recording spectrophotometer.

Cholinesterase activity was measured against a reagent blank containing an equivalent amount of serum, which had previously been incubated with 0·1 mM-DFP to inhibit all the cholinesterase activity. The difference between the absorption of the solution containing uninhibited serum and inhibited serum, after a suitable digestion time, was a measure of the cholinesterase activity. When the Hilger Uvispek was employed, the cholinesterase activity was terminated by adding DFP to the uninhibited reaction medium. Typically, 0·05 ml. of 25 mM-DFP was added to 5 ml. of buffered substrate and an appropriate volume of serum contained in a 10 ml. volumetric flask. Both inhibited and uninhibited reaction media were then diluted to 10 ml. with 50 % (w/v) ethanol and read at 414 mμ at any convenient time. Mg^{2+} ions (10 mM) did not affect the hydrolysis of *o*-nitrophenyl butyrate by serum cholinesterase. The *o*-nitrophenol absorption was calibrated in the presence of alcohols at the final concentration used in the reaction medium.

Manometric method. A modification of the method of Aldridge (1953) was used for the determination of *o*-nitrophenyl butyrate activity. Generally 0·5 ml. of an enzyme dilution was placed in the side arm and 2·5 ml. of buffered substrate was placed in the centre well. The final concentration of the reagents, exclusive of substrate, was 26 mM-$NaHCO_3$, 100 mM-NaCl and 0·2 % of acacia. The flasks were gassed with $CO_2 + N_2$ (5:95) at room temperature for 15 min. before being placed in the 37° bath. Readings were taken 2 min. after tipping and at 5 min. intervals after the first 5 min. reading. The activity was calculated from the initial velocity, and usually the readings for the first 15 min. approximated to a straight line.

RESULTS

Specificity. The substrate specificity patterns of human-serum and horse-serum cholinesterase (as judged by eserine inhibition) are compared with that of a purified cholinesterase preparation from horse serum (Strelitz, 1944, stage 2), in Fig. 2, which clearly shows the similarity of the patterns. *o*-Nitrophenyl butyrate was hydrolysed 2·4 times as rapidly as acetylcholine by human cholinesterase, compared with 1·8 times by horse-serum and purified horse-serum cholinesterases.

Within experimental limits, eserine (10 μM), and paraoxon (10 μM) inhibited the same percentage (about 97 %) of the human-serum activity hydrolysing *o*-nitrophenyl butyrate. The remaining activity was therefore attributed to A-esterases. This result was consistent with the observation of Aldridge (1953) that human serum does not contain B-esterases.

Inhibition by eserine was also determined by spectrophotometric analysis. 0·1 mM-DFP inhibited $96{\cdot}8 \pm 1{\cdot}3$ % of the enzymic hydrolysis of *o*-nitrophenyl butyrate compared with $97{\cdot}4 \pm 1{\cdot}7$ %

inhibition by 0·03 mM-eserine. The eserine pI_{50} with *o*-nitrophenyl butyrate was 8·1.

These results, and the similarity of the substrate specificity patterns of human-serum and horse-serum cholinesterases, suggested that in human serum cholinesterase was responsible for most of the enzymic hydrolysis of *o*-nitrophenyl butyrate.

The cholinesterase and A-esterase activities of different human sera towards *o*-nitrophenyl butyrate were compared, and the results are given in Table 1. Although the activities varied widely between different sera, in the serum with the lowest cholinesterase to A-esterase ratio cholinesterase was responsible for 96·3 % of the activity.

The cholinesterase and A-esterase specificity towards a number of nitrophenyl esters is compared in Table 2. These results suggested that A-esterase was most active towards *para*-orientated nitro esters substituted with acetate, whereas cholinesterase was more specific towards *ortho* esters and butyrates. The possibility of selecting a nitrophenyl ester with an orientation and substitution reasonably specific for any esterase to be determined was also suggested by these results. For example, whereas cholinesterase was responsible for 97 % of the *o*-nitrophenyl butyrate hydrolysis, A-esterases accounted for about 80 % of the *p*-nitrophenyl acetate hydrolysis under similar conditions.

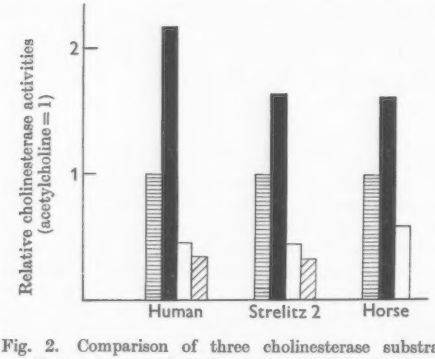

Fig. 2. Comparison of three cholinesterase substrate specificity patterns; human serum, purified preparation (Strelitz, 1944, stage 2) from horse serum, and horse serum. Activities were determined manometrically at 37° and pH 7·4. The substrate concentrations were: acetylcholine, 30 mM (▤); *o*-nitrophenyl butyrate, 4·2 mM (■); tributyrin, 16 mM (▨); *p*-nitrophenyl acetate, 4·2 mM (□). Final concentrations in digest medium, exclusive of inhibitors and substrate were: NaCl, 0·1 M; $NaHCO_3$, 26 mM; acacia, 0·2 %. The serum-cholinesterase activity was determined by the difference in the activity of uninhibited serum and serum inhibited by addition 30 min. previously of 60 μM-eserine. Final eserine concentration was 10 μM. The activity towards acetylcholine was for human sera, 66 ± 4, and horse sera, 81 ± 1 μl. of CO_2/ml. of serum/min.

Table 3 gives a comparison of the K_m and $V_{max.}$ values of *o*-nitrophenyl butyrate, *o*-nitrophenyl acetate, *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate obtained with human-serum cholinesterase. The specificity suggested by the relative activities towards these compounds was further confirmed by the K_m values. These results suggested that cholinesterase activity was affected more by the orientation of the nitro group than by the nature of the acyl substituent. Thus, substituting acetate for butyrate in the *ortho* compounds decreased the activity by half and raised the K_m by a factor of 2·5. Substituting *p*-nitrophenyl for *o*-nitrophenyl decreased the activity by a third and raised the K_m by a factor of almost 8.

For routine activity determinations, it is desirable to determine the activity at substrate concentrations where the activity is independent of the concentration. With some of the nitrophenyl esters this was not possible because of low solubilities and high K_m values. However, the *o*-nitrophenyl butyrate K_m of cholinesterase was sufficiently low and the *o*-nitrophenyl butyrate solubility sufficiently high for this condition to be easily realized.

Stability of nitrophenyl esters. The spontaneous hydrolysis rates of a number of nitrophenyl carboxylic esters are compared in Table 4. Spontaneous hydrolysis appears to be a second-order reaction, depending on the concentration of both the ester and the hydroxyl ion. When the hydroxyl ion concentration is constant, as in a buffered medium, the hydrolysis rate may be treated as a first-order reaction and comparison of rates made on this basis.

The *ortho*-nitrophenyl esters were from 1·5 to 3·8

Table 1. *Variation between individual sera of the cholinesterase and A-esterase activities towards o-nitrophenyl butyrate*

Each determination was made in duplicate on the DK-2 recording spectrophotometer. For cholinesterase determinations 0·010 ml. of serum was used and for A-esterase determinations 0·050 ml. of serum. Concn. of *o*-nitrophenyl butyrate was 1 mM, pH 7·6, temp. 25°.

Activity (μmoles of *o*-nitrophenol/ml. of serum/min.)		Cholinesterase / A-Esterase
Cholinesterase	A-Esterase	
2·84	0·049	58
2·69	0·087	31
2·48	0·080	31
2·97	0·053	56
2·14	0·070	31
1·98	0·033	60
2·80	0·105	27
2·80	—	—
Average ± S.D.		
2·59 ± 0·36	0·068 ± 0·025	42 ± 15

Table 2. *Comparison of the activities of human-serum cholinesterase and A-esterases toward nitrophenyl esters*

The activity was determined with 1 mM-substrates at pH 7·6 in 0·05 M-phosphate buffer at 25°, with the DK-2 spectrophotometer according to the method in Experimental section. The *p*-nitrophenol liberated was followed at 400 mμ. For cholinesterase activities 0·010 ml. of serum was used; for A-esterase activities 0·050 ml. of DFP-treated serum was used. An equivalent amount of pre-boiled serum was added to the A-esterase determination reagent blank.

Substrate	Activity (μmoles of nitrophenol liberated/ml. of serum/min.) Cholinesterase	A-Esterase	Cholinesterase / A-Esterase
o-Nitrophenyl butyrate	2·59*	0·068*	42
o-Nitrophenyl acetate	1·50†	0·78†	1·9
p-Nitrophenyl butyrate	0·66†	0·27†	2·41
p-Nitrophenyl acetate	0·32†	1·04†	0·31

* Average of results from Table 1.
† Pooled sera of four individuals.

Table 3. *Comparison of the human-serum cholinesterase $V_{max.}$ values and K_m values of four nitrophenyl esters*

The activities were determined with the Beckman DK-2 recording spectrophotometer at 25°, pH 7·6 in 0·05 M-phosphate buffer. Typically, five concentrations (1, 0·33, 0·2, 0·15 and 0·1 mM) of substrate were used to determine a K_m. The Dixon (1953) modification of the Lineweaver & Burk (1934) plot was used. Usually 0·010 ml. of serum was used in 5 ml. of buffered substrate. The reagent blank was identical with the activity solution, except that the serum used had been previously incubated with 0·1 mM-DFP.

Substrate	$V_{max.}$ (μmoles of nitrophenol/ml. of serum/min.)	K_m (mM)	Activity ratio (*o*-Nitrophenyl butyrate = 1)
o-Nitrophenyl butyrate	3·32	0·106	1
o-Nitrophenyl acetate	1·67	0·25	0·50
p-Nitrophenyl butyrate	1·16	0·80	0·35
p-Nitrophenyl acetate	0·60	1·08	0·18

times as stable as the corresponding *para* esters. The order of decreasing stability with respect to acyl substituent was butyrate > propioniate > acetate. For the purposes of routine activity determination, it was convenient to have a compound of reasonable stability at pH 7·6 in order to decrease possible reagent-blank errors to negligible proportions. From this point of view, *o*-nitrophenyl butyrate was the most suitable ester examined, with a half-life of 3 days under the conditions used.

In addition to spontaneous non-enzymic hydrolysis of these esters, there is a non-enzymic hydrolysis caused by the serum proteins which must also be considered when evaluating enzymic hydrolysis. As shown in Table 5, this hydrolysis was not proportional to the amount of serum used, nor was it linear with time. The rates given in Table 5 are initial rates, but the total hydrolysis in the time used for routine analysis (30 min.) was less than one-third of that suggested by the initial rate. When determinations were made with the DK-2 ratio recording spectrophotometer, the protein non-enzymic hydrolysis (together with the other non-enzymic and non-cholinesterase enzymic activities) were automatically cancelled by using a suitable reference blank. With the routine procedure, the non-enzymic serum-protein hydrolysis was decreased to negligible portions (Table 6).

Effect of butanol. Butan-1-ol activated the cholinesterase hydrolysis of *o*-nitrophenyl butyrate and inhibited the barely significant A-esterase activity. In the presence of 5% (v/v) butanol, all the measurable enzymic hydrolysis of *o*-nitrophenyl butyrate by human serum was attributable to cholinesterase. With mM-*o*-nitrophenyl butyrate, 5% (v/v) butanol increased the activity by a factor of 3·08 ± 0·21 as judged by duplicate determinations of seven different sera. The activity increased, from an average of 2·56 to 7·89 μmoles of nitrophenol liberated/ml. of serum/min., in the presence of 5% (v/v) butanol. The increase in activity was proportional to butanol concentration (Main, 1961). K_m increased from 0·11 mM in the absence of butanol to 0·46 mM in the presence of 5% (v/v) butanol. With the volume of serum used for routine determinations of *o*-nitrophenyl butyrate (0·004 ml.), the

Table 4. *Non-enzymic hydrolysis rates of various nitrophenyl carboxylic esters*

The initial concentration of each ester was 1 mM. Most solutions were prepared by diluting 0·20 ml. of a 0·50M-stock solution of ester in methanol to 100 ml. with 0·05M-phosphate buffer. With *p*-nitrophenyl acetate, 1·0 ml. of a 0·10M-methanol solution of *p*-nitrophenyl acetate was diluted to 100 ml. with buffer. The hydrolysis was followed with a DK-2 recording spectrophotometer over appropriate periods of time. The half-life $t_{\frac{1}{2}}$ was calculated assuming the hydrolysis was a pseudo first-order reaction. Temp., 25°; pH 7·6.

Ester	$t_{\frac{1}{2}}$ (min.)	$10^4 \times k^{-1}$ (min.)
o-Nitrophenyl butyrate	4500	1·5
o-Nitrophenyl propionate	1300	5·3
o-Nitrophenyl acetate	730	9·4
p-Nitrophenyl butyrate	1300	5·3
p-Nitrophenyl propionate	570	12
p-Nitrophenyl acetate	480	14

Table 5. *Non-enzymic hydrolysis of* o*-nitrophenyl butyrate and* p*-nitrophenyl acetate by previously acidified and boiled human serum*

A 1:10 dilution of human serum was acidified with 2N-acetic acid to pH 3·5 and was then placed in boiling water for 10 min. After cooling, the pH of the serum dilution was adjusted with 2N-NaOH to pH 7·3. The hydrolysis rates were determined with mM-substrate concentration at 25° and pH 7·6 on the DK-2 recording spectrophotometer. The control blanks were identical with the unknown samples except that no boiled serum was added.

Ester	Boiled serum used (ml.)	Hydrolysis, initial rate (μmoles of nitrophenol/ml. of serum/min.)
p-Nitrophenyl acetate	0·01	0·33
o-Nitrophenyl butyrate	0·01	0·26
o-Nitrophenyl butyrate	0·02	0·21
o-Nitrophenyl butyrate	0·05	0·17

Table 6. *Reproducibility of the method for determining human-serum cholinesterase with* o*-nitrophenyl butyrate*

The cholinesterase activities of a number of different human sera were determined at 25° according to the procedure in the text. Two different serum dilutions were made with each serum. The activity of each dilution was determined in duplicate, with a different preparation of buffered substrate. These determinations were made on the same day. The average activity obtained for this day is compared with that determined on a previous day to illustrate the day to day reproducibility. The *o*-nitrophenyl butyrate hydrolysis by factors other than cholinesterase was determined by first adding 0·05 ml. of 25 mM-DFP to 5 ml. of substrate solution and then adding the serum dilution. Otherwise the procedure was the same as that for determining total activity. S.D. of the duplicates with serum dilution A, ±2·5%; S.D. of the duplicates with serum dilution B, ±1·5%; S.D. between average of serum dilutions A and B, ±1·8%; S.D. between Day 1 and Day 2 results, ±1·6%; non-cholinesterase hydrolysis was 0·7%, equivalent to extinction of 0·0026±0·0027.

Extinction Serum dilution A		Extinction Serum dilution B		Non-cholinesterase hydrolysis	Activity (μmoles of nitrophenol/ml. of serum/min.) Day 1 (average of 4)	Day 2 (average of 2)
1	2	1	2			
0·334	0·340	0·335	0·332	0·003	6·92	6·96
0·420	—	0·413	0·405	0·004	8·55	8·38
0·402	0·414	0·405	0·419	0·000	8·44	8·45
0·420	—	0·413	0·410	0·001	8·56	8·14
0·300	0·295	0·315	0·295	0·002	6·62	6·77
0·401	0·376	0·362	0·359	0·002	7·72	7·40
0·430	0·405	0·414	0·410	0·008	8·55	8·73

activity was proportional to time over the entire 30 min. digestion period when the final *o*-nitrophenyl butyrate concentration was 1·25 mM, but deviated slightly toward the end in the presence of a 0·83 mM-solution. The presence of the butanol appeared to increase the *o*-nitrophenyl butyrate solubility so that 1·5 mM concentrations were readily obtained. The activity was independent of the *o*-nitrophenyl butyrate concentration at 1·25 mM, so that variations in the concentration (1·3±0·3 mM) did not result in measurable loss of accuracy.

Determinations were slightly more reproducible if the enzyme was diluted in buffer containing 5% (v/v) butanol and 0·1% of acacia before being added to the substrate.

Effect of pH. The effect of pH on the cholinesterase activity towards *o*-nitrophenyl butyrate was determined in the presence and absence of butanol with the results shown in Fig. 3. The activity was essentially independent of the pH between pH 7·2 and 8·8 in the presence of butanol. With no butanol the activity was independent of the pH above a pH of 7·4, but the plateau was extended beyond the range found in the presence of butanol. A pH of 7·6 was chosen for most activity determinations, because of the stability of the *o*-nitrophenyl butyrate (Table 4). Small fluctuations

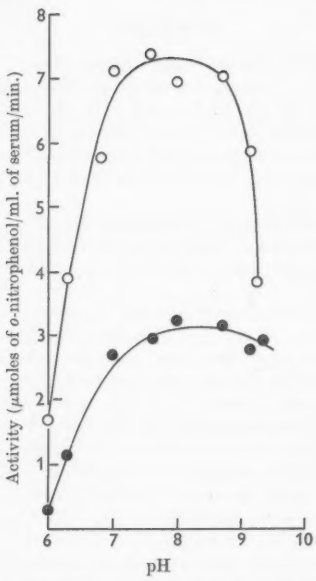

Fig. 3. Hydrolysis of *o*-nitrophenyl butyrate by human-serum cholinesterase as a function of pH in the presence of 4·2% of butanol (○) and without butanol (●). Activities were determined at 25°; 0·05M-phosphate buffers were used to cover the range from pH 6 to 7·8. From pH 8·0 to 9·25, 0·05M-borate buffers were used. The Hilger Uvispek was employed for all determinations. From 0·02 to 0·004 ml. of serum was used, depending on conditions. Digest time varied from 10 to 30 min. The *o*-nitrophenyl butyrate concentration was 1·0 mM.

in pH in this region would not appreciably affect the activity.

The cholinesterase activity toward *o*-nitrophenyl butyrate was proportional, within the limits of experimental error, to the amount of serum used over the range of volumes tested (0·001–0·100 ml.) at pH 7·6 and 25°.

Routine determination of human-plasma-cholinesterase activity

Buffered substrate. 0·5M-*o*-Nitrophenyl butyrate stock solution in methanol (0·3 ml.) was diluted to 100 ml. with 5% (v/v) butan-1-ol, pH 7·6, and 50 mM-phosphate buffer, according to the method described in the Experimental section. The concentration of *o*-nitrophenyl butyrate was 1·5 mM.

Plasma dilution. Blood was collected from finger punctures in capillaries as previously described. Plasma (0·02 ml.) was diluted to 5 ml. in ice-cold 5% butanol (v/v at 25°), pH 7·6 and 50 mM-phosphate buffer containing 0·1% acacia. Sahli blood pipettes calibrated 'to contain' were used to pipette the plasma.

The enzyme dilution was stable for at least 30 min. at room temperature. If delays greater than this were necessary before its use, preparation and storage in ice-cold buffers was advisable.

Procedure. Buffered substrate (5 ml.) was pipetted into two 10 ml. volumetric flasks (A and B). About 0·05 ml. (1 drop) of 25 mM-DFP was added to flask B and the flasks were placed in a constant-temperature bath at 25°. Plasma dilution (1·0 ml. containing 0·004 ml. of plasma) was added to flask A and B in that order to avoid DFP contamination of A. After 30 min., 0·05 ml. (1 drop) of 25 mM-DFP was added to flask A and both A and B were made up to volume with 50% (v/v) ethanol. The colour was read at 414 mμ with the contents of flask B as a reagent blank.

An example of the precision of the procedure is given in Table 6 where the duplicate determinations of a single plasma dilution are compared with the duplicate determinations of another dilution. In addition, the day-to-day precision is given. A measure of the non-enzymic plasma-protein hydrolysis is also given. This was obtained by measuring the difference in extinction between flask B and the contents of a flask identical with B, except that it had not been digested 30 min. before the addition of 50% (v/v) ethanol. The serum-protein non-enzymic hydrolysis was about 0·7% of the enzymic hydrolysis, but was so low as to be barely within the range of the spectrophotometer. This error was considerably less than the precision of the method (about ±2·5%).

Each determination required an estimated 2–3 min. manipulation. The activities were calculated in terms of μmoles of *o*-nitrophenol liberated/ml. of plasma/min. at pH 7·6 and 25°.

DISCUSSION

Huggins & Lapides (1947) described the use of *p*-nitrophenyl carboxylic esters as substrates in sensitive methods of determining activities of serum esterases. The nature and substrate specificity patterns of these esterases in sera and tissues has since been further clarified by a number of workers (for example Aldridge, 1953; Mounter & Whittaker, 1953; Bergmann, Segal & Rimon, 1957; Main, 1960).

Aldridge (1953) divided serum esterases into A and B classifications, neither of which included cholinesterases. A-Esterases hydrolysed acetate in preference to butyrate esters and were not inhibited by organophosphorus compounds. B-Esterases had the reverse specificity and, like cholinesterases, were inhibited by organophosphorus compounds. Cholinesterases and B-esterases were differentiated

by the inhibitor eserine. Main (1960) demonstrated that the orientation of the nitro group on the nitrophenyl part of these esters also influenced the activity. The A-esterases of sheep serum, for example, hydrolysed *m*-nitrophenyl acetate more rapidly than *p*-nitrophenyl acetate. The activity towards *o*-nitrophenyl acetate was only one-twenty-seventh of that towards the *meta*-isomer.

In the present work it has been shown that the substrate specificity of human-serum A-esterases is similar to that of sheep serum A-esterases. In contrast, human-serum cholinesterases hydrolysed *ortho*-isomers more rapidly than *para*-isomers. The results suggested that the nitro orientation, more than the nature of the acyl substituent, influenced the cholinesterase activity, but that the reverse was true for the A-esterases (Table 2).

In human serum, B-esterases are absent (Aldridge, 1953). Consequently, only the A-esterases and cholinesterase were concerned in the hydrolysis of esters. The present method was developed by applying and further extending this knowledge about the acyl and the isomeric nitro specificity of these chromogenic substrates.

For routine analysis, colorimetric methods generally have the advantage of sensitivity, rapidity and relative simplicity of manipulation over manometric, titrimetric and electrometric methods. In the past, colorimetric methods for the determination of serum-cholinesterase activity have not found wide acceptance for routine analysis. The phenyl benzoate method of Gomori (1949), for example, suffered from the disadvantage of low phenyl benzoate solubility and a relatively high K_m value. As a result, the activity was a critical function of the phenyl benzoate as well as of the enzyme concentration. In the author's experience, reproduction of a given substrate concentration was difficult to achieve, and consequently poor agreement was frequently observed between activities measured on different days. The β-carbonaphthoxycholine method of Ravin, Tsou & Seligman (1951) has the disadvantages of a low substrate solubility (0·12 mM) and a tedious diazotization and extraction step. In the present method activity was independent, within fairly wide limits, of the substrate concentration. In human serum or plasma, the substrate was highly specific and sensitive, particularly in the presence of butanol. Since *o*-nitrophenol could be measured directly, no colour-developing procedure was necessary. As a result, the method requires only 2 or 3 min. of manipulation per determination and is reasonably accurate (precision within $\pm 2{\cdot}5\%$).

SUMMARY

1. Human-serum and horse-serum cholinesterases hydrolysed *o*-nitrophenyl butyrate about twice as rapidly as acetylcholine. Human-serum cholinesterase had a greater affinity (K_m 0·11 mM), and greater activity towards *o*-nitrophenyl butyrate than toward any other nitrophenyl carboxylic ester tested.

2. Human-serum cholinesterase hydrolysed *ortho*-nitrophenyl carboxylic esters more rapidly than the *para* esters. A-Esterases had the reverse specificity.

3. In human serum, the cholinesterase was responsible for not less than 96% of the enzymic hydrolysis of *o*-nitrophenyl butyrate. A-Esterases accounted for the remaining activity.

4. In the presence of 5% (v/v) butan-1-ol, cholinesterase-catalysed hydrolysis of *o*-nitrophenyl butyrate was activated threefold, whereas the A-esterase activity was completely inhibited.

5. A rapid, sensitive, colorimetric method suitable for routine analysis was developed as a consequence of these investigations. Over conveniently wide ranges, the activity was independent of both the pH and substrate concentration. The *o*-nitrophenol liberated by hydrolysis was measured directly in the reaction medium. The colour was stable. The precision of the method was $\pm 2{\cdot}5\%$; each determination required 2–3 min. manipulation.

6. A method for the synthesis and purification of *o*-nitrophenyl butyrate is described.

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Acetate Utilization in Sheep

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(*Received* 11 *May* 1960)

Acetate, an important metabolite in most species, has a unique place in ruminant metabolism. As the major product of ruminal and caecal fermentation, acetate provides much of the energy requirements of ruminant tissues. Little information is available, however, on the overall utilization of acetate in sheep. Intravenous-acetate tolerance tests in sheep (Jarrett, Potter & Filsell, 1952; Pugh & Scarisbrick, 1952; Reid, 1958) have indicated that raised concentrations of circulating acetate fall more slowly than in cats (Smyth, 1947) or dogs (Ciaranfi & Fonnesu, 1954). A serious limitation of tolerance tests is that utilization rates can be examined only at high, unphysiological and continuously varying concentrations. In the present studies an isotope-dilution technique has been used to determine overall utilization rates at normal concentration of circulating acetate. The constant-infusion technique employed was similar to that used by Steele, Wall, de Bodo & Altszuler (1956) for the measurement of glucose-utilization rates in dogs. In this technique the rate of entry of acetate into the circulation is measured, and under steady-state conditions this will equal the rate of acetate leaving the plasma, i.e. the utilization rate.

In addition, the utilization of acetate has been studied at normal and enhanced levels of blood acetate, high concentrations of circulating acetate being achieved by infusing unlabelled acetate with the ^{14}C-labelled material. Similarly, by infusing either glucose or insulin with labelled acetate, acetate-utilization rates at high and low levels of blood glucose have been measured. Comparison of the specific activity of expired carbon dioxide and plasma acetate during the infusion of [*carboxy*-^{14}C]-acetate and [*methyl*-^{14}C]acetate has allowed rough estimates to be made of the contribution of acetate to the oxidative metabolism of sheep. A preliminary account of this work has been given to The Biochemical Society (Annison & Lindsay, 1958).

MATERIALS AND METHODS

Experimental animals. Mature Clun Forest ewes were fed once daily (1000 g. of chopped hay) and trained to consume their food within 2 hr. To accustom them to the conditions of the experiments, the animals were fitted with the face masks used for respiratory CO_2 collection and placed in stocks for several hours daily. Sheep which failed to stand quietly under these conditions were rejected. In some experiments sheep with permanent rumen fistulae were used, and two sheep prepared with tracheal cannulae (Webster & Cresswell, 1957) were also available when it was necessary to exclude rumen gases from expired air. Sheep were fasted for 24 hr. before each experiment.

Experimental procedure. The general arrangement is shown in Fig. 1. Polythene catheters were inserted into both jugular veins under local anaesthesia (procaine), 2–3 hr. before the start of an experiment. The left-side catheter, which was used for the injection or infusion of labelled acetate, was inserted downwards, i.e. towards the heart, about 6–8 in. The second catheter, for the withdrawal of blood samples, was inserted upwards (3–4 in.). This procedure was adopted to avoid possible contamination of blood samples with infused material. Constant infusion was maintained by using an apparatus built in this Institute. It consisted of two stainless-steel cylinders facing one another which were alternately filled and emptied by movement of pistons on a common shaft. Valves ensured that one cylinder filled from a reservoir while the second was infusing solution into the animal. Constancy of rate of movement of the pistons was ensured by means of a Velodyne (Williams & Uttley, 1946).

Respired CO_2 was collected with a rubber face-mask at the end of which was a bifurcated Perspex tube fitted with thin rubber-flap valves. Expired air passed through an absorption train of NaOH, as shown in Fig. 2. Collections were made for 3–5 min. periods at intervals during the infusion period. The absorption train consisted of three towers (two are shown in Fig. 2) containing 20% (w/v) of NaOH (effective height of column of alkali in each tower was 5 cm., and total volume 100 ml.). Absorption of CO_2 was facilitated by dispersal of the gas mixture with sintered-glass (200–250 μ porosity) disks placed near the base of the towers (Fig. 2). Suction of the pump (Fig. 2) was arranged to match the expired air output of the animal by keeping the spirometer height constant. Further washing of the gas leaving the absorption train with $Ba(OH)_2$ (0·2N) contained in additional absorption tubes indicated that at the flow rates of gas used in the experiments approx. 5% of the expired CO_2 escaped absorption.

Urine was collected by bladder catheterization during the period of the experiment.

Heparin was used as an anti-coagulant throughout the investigation.

Chemical methods

Determination of plasma acetate. Acetate accounts for 80–90% of the volatile fatty acids of sheep blood, the remainder consisting largely of formate (Annison, 1954). The acids are not distributed equally between plasma and red cells, however, the latter containing a greater proportion of formate. Plasma formate levels rarely exceed 5% of

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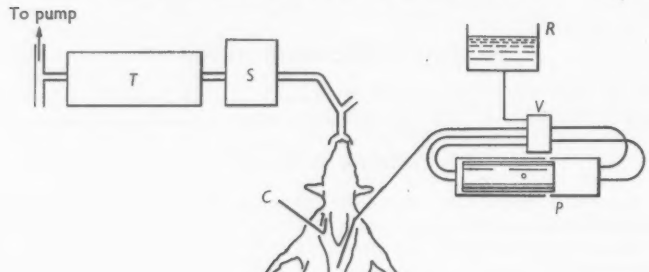

Fig. 1. General arrangement of apparatus for constant intravenous infusion of isotope into sheep. *T*, Absorption towers; *S*, spirometer; *R*, reservoir of isotope; *P*, constant infusion pump; *V*, valves; *C*, sampling catheter.

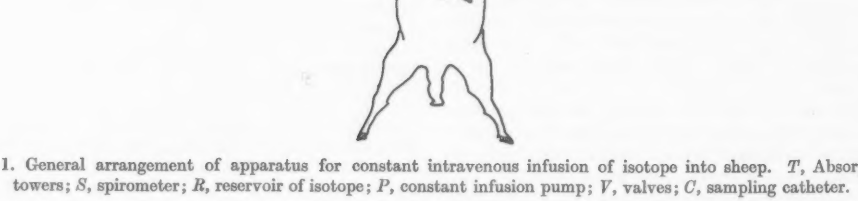

Fig. 2. Apparatus used for collecting expired CO_2. *M*, Face-mask; *F*, flap valves; *S*, spirometer; *T*, absorption tower.

the total volatile fatty acid concentrations, and steam-distillation of deproteinized plasma filtrates at pH 4·0 decreases the amount of formate in the distillates to negligible proportions. The procedure of Annison (1954) was used in the deproteinization of plasma and the concentration and distillation of filtrates, except that the solutions were adjusted to pH 4·0, and not pH 3·0, before steam-distillation. Quantitative recoveries of acetate were obtained, and the acetate content of filtrates was occasionally checked by gas–liquid chromatography (James & Martin, 1952).

Assay of radioactive acetate. To steam-distillates, after titration with 0·01 N-NaOH, were added 0·2 ml. of N-NaOH. They were concentrated by boiling to a volume of 0·5–1·0 ml. The solutions were then dried in air on aluminium planchets and assayed for radioactivity with an end-window Geiger–Müller tube. If less alkali was added to the distillates before drying, acetate was lost from the planchets on standing in air. The quantity of sodium acetate (0·5–1·5 mg.) obtained in the steam-distillates was small relative to the amount of added alkali (8 mg.). A standard curve was made by plotting counts/min. against added radio-

activity whilst maintaining the total sodium acetate concentration constant at 1·5 mg. Alkali (0·2 ml. of N-NaOH) was added to the solutions, which were concentrated and dried as in actual assays. A linear plot was obtained.

Assay of carbon dioxide in expired air. A gas-transfer apparatus similar to that described by Kornberg, Davies & Wood (1952) was used to transfer CO_2 from the alkali in the absorption train to $Ba(OH)_2$. Unchanged $Ba(OH)_2$ was titrated with 0·2N-HCl with phenolphthalein as indicator, and the precipitated $BaCO_3$ was separated by centrifuging. The precipitate was washed twice with water, then with acetone, and transferred to planchets as a slurry in acetone. After drying in air, the $BaCO_3$ was counted with an end-window Geiger–Müller tube. Counts were corrected to infinite thickness.

Blood carbon dioxide. Blood (10 ml.) was withdrawn slowly into a syringe lubricated with liquid paraffin and containing heparin (150 units) as anti-coagulant. The samples were stored at 4° in the syringes until used, when about 10 ml. of blood, the precise volume being determined by change of weight of the syringe, was added to 0·5 ml. of 0·5N-NaOH (CO_2-free) contained in a modified gas-transfer apparatus. The apparatus was evacuated and 3 ml. of 2N-lactic acid was slowly added to the blood, the liberated CO_2 being absorbed in $Ba(OH)_2$ and treated as described above.

Wet oxidation of acetate. The solutions of [*carboxy*-^{14}C]-acetate and [*methyl*-^{14}C]acetate (The Radiochemical Centre, Amersham, Bucks.) used in the infusion experiments were standardized by wet oxidation to $^{14}CO_2$ (van Slyke & Folch, 1940) which was absorbed in $Ba(OH)_2$ and assayed as $BaCO_3$. Absolute levels of radioactivity were obtained by reference to a standard source [^{14}C polymer reference source (CFP1) obtained from The Radiochemical Centre]. A portion of the standard was also converted into $BaCO_3$ by wet combustion and counted at infinite thickness.

Determination of blood glucose. Blood glucose was determined by the method of Somogyi (1952) with the colorimetric modification of Nelson (1944).

Measurement of utilization rate. The term 'utilization' has been used in this paper to mean the rate of removal of metabolite from the plasma. In practice, isotope dilution measures the rate at which a metabolite is added to the plasma. Under steady-state conditions when the concentration of, say, acetate is constant, rates of addition and removal are equal. Utilization rates (U, m-moles/min.) have been calculated from the expression $U = X/Y$, where X is rate of infusion of isotope (μc/min.), and Y is specific activity of plasma acetate (μc/m-mole).

RESULTS

Equilibration of [^{14}C]*acetate between red cells and plasma.* Plasma was used instead of whole blood in the assay of acetate to avoid interference by formate. Before adopting this procedure, however, it was necessary to establish that injected labelled acetate quickly equilibrated with the acetate in red cells. A small volume (1 ml.) of labelled acetate (20 μc) in 0·9 % sodium chloride solution was added to 50 ml. of sheep blood maintained at 37°. After mixing thoroughly, a portion of blood was removed and centrifuged to separate red cells and plasma. The haematocrit was noted. The remainder of the blood was shaken gently at 37° for 30 min., when a further portion was separated into red cells and plasma. Analysis of each of the fractions for acetate showed that the specific activity was the same in all fractions, indicating rapid equilibration of added acetate between red cells and plasma.

Half-time of injected [carboxy-^{14}C]*acetate.* The specific activity of acetate in sheep plasma was determined at intervals of 2–3 min. (accurately measured), after the injection of 100 μc of sodium [*carboxy*-^{14}C]acetate (40 μmoles) in 5 ml. of solution. The rapid turnover of acetate was indicated by the results shown in Fig. 3, where specific activity was plotted against time on a semi-logarithmic scale. The slope was clearly not fixed with any great precision but a half-life of 3–4 min. was indicated. From the intercept, the apparent volume of distribution was approx. 70 l. This is about 1·5 l./kg. body wt.

Utilization rates of acetate. Utilization rates of acetate at normal and enhanced levels of plasma acetate were conveniently determined by the constant-infusion method during the same experiment. A solution of labelled acetate containing negligible amounts of carrier acetate was infused for 3 hr., as Tables 1 and 2 show; the specific activity of plasma acetate had reached a constant level within 2 hr. A solution containing the same amount of radioactivity per unit volume, but in addition a high concentration of unlabelled sodium acetate, was now infused at the same rate, care being taken to limit the interruption of isotope inflow to 5–10 sec. Steady-state conditions of plasma acetate were usually attained within 30–50 min. The short half-time of acetate found by single injection experiments made it unnecessary to use an initial priming dose of acetate, a technique found useful by Steele *et al.* (1956) in the establishment of constant specific activity of plasma glucose in the dog.

In a typical experiment with a 44·0 kg. ewe, [*carboxy*-^{14}C]acetate was infused for 167 min. at an inflow rate of 0·494 μc/min. (2 μmoles/min.). For the next 150 min. the same infusion of [*carboxy*-^{14}C]-acetate was maintained, together with carrier acetate at 1·31 m-moles/min. Plasma acetate concentrations remained constant during the infusion of essentially carrier-free [*carboxy*-^{14}C]acetate at 0·25 m-mole/l. but gradually increased during the second period to 2·20 m-moles/l. (Table 1). The mean utilization rate during the first period (0·51 m-mole/min.) increased to 1·83 m-moles/min. when steady-state conditions had been reached at the raised level of plasma acetate. The increase in mean utilization rate (1·83 − 0·51 = 1·32 m-moles/min.) was in excellent agreement with the rate of infusion of carrier acetate (1·31 m-moles/min.).

When methyl-labelled acetate was used in a similar experiment with a 27·1 kg. sheep, inflow rate 0·506 μc/min. during first period, and carrier acetate infused at 1·30 m-moles/min. during the second period, the results shown in Table 2 were obtained. The agreement between infusion rate and increase in utilization rate was again good.

Effect of glucose on acetate utilization rates. The effects of raised or diminished levels of blood glucose on acetate utilization rates were examined

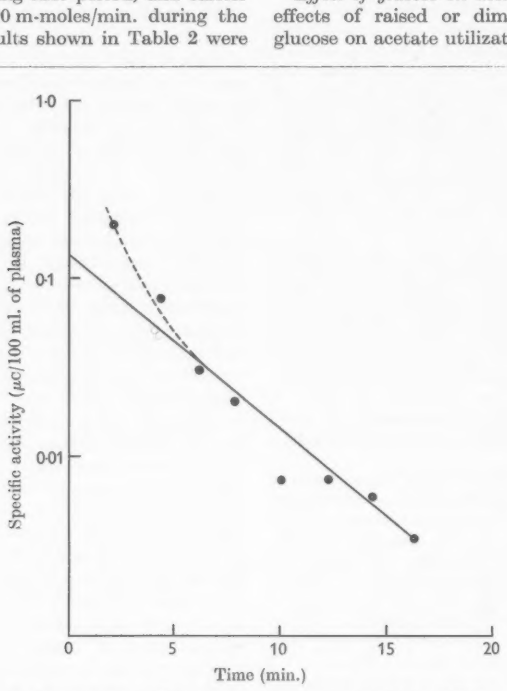

Fig. 3. Radioactivity in plasma acetate after a single intravenous injection of 100 μc of [*carboxy*-^{14}C]acetate. Concn. of acetate, 0·47 m-mole/l. throughout the period.

Table 1. *Measurement of utilization rate of acetate at normal levels of plasma acetate* (0–167 *min.*), *and at high acetate concentrations* (208–322 *min.*) *by infusion of sodium* [carboxy-^{14}C]*acetate*

Labelled acetate (0·494 μc/min.) was infused for 330 min. Carrier acetate was infused from 0 to 167 min. and from 167 to 317 min. at the rates 2 μmoles and 1·31 m-moles/min. respectively. Mean utilization rates: 102–167 min. = 0·51 m-mole/min.; 249–322 min. = 1·83 m-moles/min. The ewe weighed 44 kg.

Time after start of infusion (min.)	Plasma acetate (m-moles/l.)	Specific activity of acetate (μmc/m-mole)	Specific activity of blood CO_2 (μmc/m-mole)	Blood glucose (mg./100 ml.)
23	—	—	—	37·0
102	0·25	775	28·2	42·0
130	0·25	970	33·0	—
145	0·25	1000	35·0	—
155	0·25	987	36·8	43·0
167	0·25	945	34·6	—
208	—	—	43·6	32
240	—	—	39·6	—
249	2·19	277	—	—
264	—	—	40·7	—
280	2·22	286	—	—
285	—	—	37·2	—
297	2·20	278	—	38
304	2·22	265	46·3	—
322	2·20	250	53·1	—

by infusing carboxyl-labelled acetate at a constant rate (*a*) alone, (*b*) with added glucose and (*c*) with added insulin. In each of the three periods of the experiment sufficient time was allowed for steady-state conditions to be obtained. The results of a typical experiment are shown in Table 3. A moderate elevation of blood glucose levels increased acetate utilization. Infusion of insulin lowered blood-sugar levels, and depressed acetate utilization. At very high levels of blood glucose, acetate utilization was depressed, as the results of an experiment shown in Table 4 indicate. In this experiment infusion of insulin again depressed acetate utilization.

Specific activity of expired carbon dioxide. Expired air in ruminants may contain carbon dioxide eructated from the rumen in addition to expired carbon dioxide. To eliminate the contribution of ruminal carbon dioxide, sheep prepared with tracheal cannulae were used in early experiments. Later it was found (Fig. 4*A*) that there was close agreement between the specific activities of carbon dioxide from expired air and in venous (jugular) blood during infusion of $NaH^{14}CO_3$. This allowed the collection of expired carbon dioxide to be dispensed with in experiments in which only the specific activity of carbon dioxide, but not total output, was required. However, more recent experiments have shown that the agreement of blood and expired air specific activities is not found when labelled acetate is used. Fig. 5 shows that on average (jugular) blood carbon dioxide has a specific activity about 15% lower than that of expired air. Since expired air is of higher specific activity than blood carbon dioxide, this can hardly be due to dilution of expired air with eructated (inactive) rumen gas.

In order to interpret the relationships between

Table 2. *Measurement of utilization rates of acetate at normal* (0–167 *min.*), *and raised* (167–330 *min.*) *levels of plasma acetate by the infusion of sodium* [methyl-^{14}C]*acetate*

Labelled acetate (0·506 μc/min.) was infused for 330 min. Carrier acetate was infused from 0 to 167 min. and from 167 to 330 min. at the rates 2 μmoles and 1·30 m-moles/min. respectively. Mean utilization rates (*a*) (124–157 min.) 0·43 m-mole/min., (*b*) (207–330 min.) 1·80 m-moles/min. The ewe weighed 27·1 kg.

Time after start of infusion (min.)	Plasma acetate (m-moles/l.)	Specific activity of acetate (μmc/m-mole)	Specific activity of blood CO_2 (μmc/m-mole)	Blood glucose (mg./100 ml.)
124	1·09	1090	—	57
137	1·01	1290	20·6	—
146	1·03	1250	22·0	—
157	1·01	1110	22·8	55
207	—	—	25·6	51
243	—	—	28·8	—
275	—	—	26·4	—
285	2·34	235	26·0	—
303	2·45	229	25·4	—
314	2·32	289	28·3	—
330	2·35	268	27·4	55

Table 3. *Measurement of utilization rates of acetate in a* 42·0 *kg. sheep*

Rates were measured at (*a*) normal levels of blood glucose (0–137 min.), (*b*) raised levels produced by the infusion of glucose (0·067 g./min.) over period of 137–284 min., and (*c*) depressed glucose levels resulting from the infusion of insulin (0·23 unit/min.) over a period of 284–412 min. Sodium [*carboxy*-^{14}C]acetate was infused continuously throughout all three periods at 0·580 μc/min.

Time after start of infusion (min.)	Plasma acetate (m-mole/l.)	Specific activity of acetate (μmc/m-mole)	Blood glucose (mg./100 ml.)	Mean acetate utilization rate (m-mole/min.)
83	0·77	160	57	
98	0·78	181	52	3·58
120	0·78	163	48	
137	0·79	144	48	
248	0·78	100	73	
265	0·79	143	71	4·93
281	0·79	128	64	
284	0·80	98	64	
375	0·91	159	24	
385	0·92	203	23	2·96
395	0·90	233	25	
412	0·91	203	25	

Table 4. *Measurement of utilization rates of acetate in a 31·5 kg. sheep*

Rates are measured at (*a*) normal levels of blood glucose (0–120 min.), (*b*) raised levels produced by the infusion of glucose (0·17 g./min.) (120–247 min.), and (*c*) depressed glucose levels (insulin infused at 0·54 unit/ml.) (247–363 min.). Sodium [*methyl*-^{14}C]acetate was infused at 0·536 μc/min. throughout.

Time after start of infusion (min.)	Plasma acetate (m-mole/l.)	Specific activity of acetate (μmc/m-mole)	Blood glucose (mg./100 ml.)	Mean acetate utilization rate (m-mole/min.)
90	0·59	168	60	
98	0·50	214	62	2·93
111	0·64	186	62	
120	0·56	169	63	
218	0·70	188	170	
230	0·63	250	178	2·29
241	0·57	269	178	
247	0·54	247	173	
340	0·52	235	17	
350	0·61	295	16	1·94
360	0·56	299	14	
363	0·58	283	18	

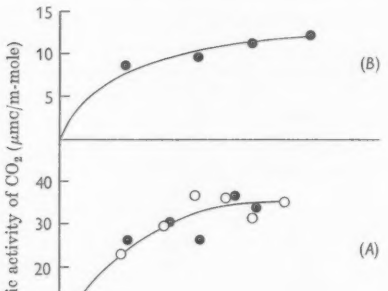

Fig. 4. Changes in specific activity of $^{14}CO_2$ during continuous intravenous infusion of $NaH^{14}CO_3$. *A*, Ewe (35 kg.) infused at the rate of 0·465 μc/min.; *B*, wether (29 kg.) infused at the rate of 0·320 μc/min. Carrier bicarbonate was negligible in both. ●, Expired-air CO_2; ○, jugular-blood CO_2.

Fig. 5. Changes in specific activity of CO_2 during continuous intravenous infusion of [*carboxy*-^{14}C]acetate. Infusion was at the rate of 0·555 μc/min. with negligible carrier acetate. The ewe weighed 70 kg. ○, Expired-air CO_2; ●, jugular-blood CO_2.

the specific activities of expired carbon dioxide and infused [^{14}C]acetate, it was necessary to investigate the rate of mixing of labelled carbon dioxide with the carbon dioxide pool of the animal. The results shown in Fig. 4 (*A* and *B*) were obtained when labelled bicarbonate was infused intravenously at a constant rate (0·465 μc/min.). Infused labelled carbon dioxide was largely equilibrated with the exchangeable carbon dioxide of the body within 2–3 hr. from the start of the infusion.

The specific activities of blood carbon dioxide shown in Tables 1 and 2 were used to obtain approximate estimates of the fractional contribution of acetate to the carbon dioxide output. This was given by the ratio specific activity of CO_2/0·5 of specific activity of plasma acetate, where the factor 0·5 was used because it was assumed that both acetate C atoms contributed to carbon dioxide, although only one was labelled. Thus in Table 1, with mean values of the specific activities of acetate and carbon dioxide over the period 130–167 min., the ratio was 0·07, and for the period 304–322 min., 0·4. That is, with the plasma acetate concentration 0·25 m-mole/l., about 7 % of expired carbon dioxide may be considered as derived directly from acetate; with the plasma acetate

concentration 2·2 m-moles/l., the value was about 40 %.

Corresponding values when methyl-labelled acetate was used (Table 2) were 3·5 % when the plasma acetate level was 1·1 m-moles/l. and 20 % at 2·3–2·5 m-moles/l. These values are about 15 % low for reasons discussed above. Corrected values are therefore 8·1 and 46 % for carboxyl-labelled acetate and 4·0 and 23 % for methyl-labelled acetate.

Excretion of acetate in urine. Urine was collected by bladder catheterization during the course of an infusion experiment. Acetate was isolated from urine by steam-distillation (Annison, 1954) and assayed for radioactivity. In a typical experiment in which 109 μc of [*carboxy*-^{14}C]acetate was infused in 120 min., 95 ml. of urine was excreted containing labelled acetate at the concentration of 1·07 μmoles, 0·41 μmc/ml. It was concluded that losses of acetate by urinary excretion were of negligible proportions.

DISCUSSION

Utilization of glucose in the dog has been extensively studied by the method of isotope dilution, both by single injection (e.g. Baker, Shipley, Clark & Incefy, 1959; Wrenshall, 1955) and by constant-infusion techniques (Searle, Strisower & Chaikoff, 1956; Steele *et al.* 1956) and the limitations of these techniques are now fairly well understood (e.g. Wrenshall & Hetenyi, 1959). For acetate, the use of a single-injection system is even more restricted than for glucose. Such a method requires that the rate of mixing be rapid compared with the rate of utilization. The short half-life of acetate (2–3 min.), suggests that this requirement is not met, and this is also supported by the improbably large value for the volume of distribution of acetate (70 l.). Such an enhanced value would be found if there were significant loss of injected radioactivity before mixing was completed. Constant infusion of labelled acetate eliminates this difficulty, sufficient time being allowed before samples were taken for a steady state to be reached between infusion of label and utilization. Preliminary experiments suggested that this steady state was reached within 20–30 min. from the beginning of infusion. However, for the experiments reported, at least 1 hr. elapsed before samples were taken, and the constancy of specific activity supports the view that a steady state had indeed been reached.

Acetate entering the plasma may be considered as coming from two sources, (*a*) from the gastro-intestinal tract (mainly from the rumen), and (*b*) from endogenous metabolism (from the liver, fat depots, etc.). Some preliminary experiments by one of the authors (D.B.L.) suggest that endogenous acetate production is about 1–1·5 m-moles/min. in a fed animal. It is probable therefore that the major part of the acetate entering the blood in the initial experiments shown in Tables 1 and 3 was endogenous. In the experiments reported in Tables 3 and 4, rumen acetate presumably made a significant contribution to the total acetate inflow, up to 2 m-moles/min. This may be compared with a value of 1–1·5 m-moles/min., which may be inferred from data of Annison, Hill & Lewis (1957) obtained by an entirely different method. There is no obvious reason why there should be a difference between the initial rates in Tables 1 and 2 and Tables 3 and 4. In all cases the animals had not been fed for 24 hr.

The distinction between acetate entry (production rate) and removal (utilization rate) would seem to be important in considering the effects of glucose and insulin on acetate utilization (Tables 3 and 4). The results suggest that this was reduced by infusion of insulin, or by considerably raised levels of blood sugar (which would, of course, stimulate endogenous insulin output), whereas utilization rates were increased by small rises in blood sugar. Since these treatments altered the specific activity of the plasma acetate, they must have influenced acetate entry into the blood. However, the outflow of acetate must also have been affected, otherwise the concentration would have changed. This is not unexpected since Reid (1950) has shown that utilization of acetate in the sheep is proportional to its concentration. Thus changes in concentration due to changes in rate of entry of acetate will tend to be self-limiting. An effect of insulin or glucose infusion on acetate production by the rumen is unlikely and it seems more probable that the effect is on the endogenous output of acetate.

The measurement of acetate utilization in the sheep in the period immediately after feeding would be of considerable interest. Ruminal fermentation and acetate production rise to a peak 3–6 hr. after feeding, however (cf. Annison & Lewis, 1959), and then decline for a further 12–18 hr. Reasonably constant levels of plasma acetate are only obtained after a day's fasting, and utilization rates can be measured by the isotope dilution method only when the rate of acetate entry to the system is fairly constant, i.e. under conditions of constant specific activity of plasma acetate. In any one animal, however, the measurement of acetate utilization at various concentrations of acetate achieved by the infusion of unlabelled acetate should allow acetate entry at any instant to be deduced subsequently by reference only to the plasma acetate concentration, provided that the experimental conditions are unchanged. In the experiment reported in Table 1, utilization rates were measured in the starved animal (plasma acetate, 0·25 m-mole/l.; utilization rate, U, 0·51 m-mole/min.) and at plasma acetate concentrations

similar to those observed 3–6 hr. after feeding (plasma acetate, 2·2 m-moles/l.; U, 1·83 m-moles/min.).

Estimation of the contribution of acetate to the oxidative metabolism of the sheep must be regarded as approximate, since the interpretation of carbon dioxide specific-activity curves is subject to many uncertainties. Difficulty arises because of the existence of a large pool of body carbon dioxide with which metabolically produced carbon dioxide has to mix to a variable extent before it is expired. The rate of mixing appears to be comparable with the rate of production of metabolic carbon dioxide (Fig. 4).

We have interpreted the ratio, carbon dioxide specific activity/plasma acetate specific activity, as indicating the fraction of total carbon dioxide produced which is contributed by the direct oxidation of acetate. The validity of this depends on at least two important factors: (1) that mixing of labelled carbon dioxide with body carbon dioxide is complete; (2) that the conversion of acetate into other metabolites and their subsequent oxidation are negligible compared with the direct oxidation of acetate. To some extent the two assumptions are self-cancelling. Secondary oxidation of acetate will be negligible initially but increasingly important as the time of infusion continues; whereas mixing errors become less important as infusion continues. The shape of curve A (Fig. 4) suggested that mixing errors were small after 2–3 hr., but in another experiment (curve B, Fig. 4) a stable plateau was not reached even after infusion of bicarbonate for 4 hr., although the value at 2–3 hr. was 80 % of the 4 hr. value. These variations may have been due to differences in the size and rate of mixing of the carbon dioxide pool, perhaps because there is a variable rate of mixing between the rumen carbon dioxide pool and the body carbon dioxide pool.

It has been shown in the course of another investigation that the specific activities of plasma glucose, and of the free fatty acid and triglyceride fractions of plasma, after the constant infusion of [*carboxy*-^{14}C]acetate for 4 hr. were less than 1 % of the specific activity of plasma acetate. The data of Harper, Neal & Hlavacek (1953) suggested that, in the dog, labelling of tissue lipids was of the same order as that of plasma lipids. It seems likely, therefore, that even if lipids or glucose or both were oxidized as rapidly as acetate, about 95 % of the ^{14}C in expired carbon dioxide up to 3 hr. after infusion will be derived from the oxidation of acetate. Mixing errors are probably more serious than secondary-oxidation errors.

The smaller amount of carbon dioxide expired from methyl-labelled, compared with carboxyl-labelled acetate is in line with observations on other animals (see Weinman, Strisower & Chaikoff, 1957). As these authors showed, if carbon dioxide arises principally from the tricarboxylic acid cycle, then methyl- and carboxyl-labelled acetate would give rise to equal amounts of labelled carbon dioxide when there is no turnover of di- and tricarboxylic acids. When there is such a turnover, more methyl- than carboxyl-carbon is carried away into synthetic reactions, and less appears as carbon dioxide. Taking account of this, our results would suggest that in the starved sheep (i.e. 24 hr. after feeding) about 6 % of the expired carbon dioxide may be derived from the carbon of acetate, but that at plasma acetate levels found in sheep after feeding 35 % or more may be so derived.

SUMMARY

1. Rates of utilization and oxidation of acetate have been studied in sheep with [^{14}C]acetate.

2. The specific activity of acetate in red cells and plasma, after the addition of labelled acetate *in vitro*, was the same within 30 sec. of addition as after 30 min.

3. Estimation of the utilization rate by making a single injection of isotope and following the change of specific activity with time was found unsatisfactory.

4. Satisfactory results were obtained by constantly infusing isotope until the specific activity of plasma acetate was constant. Fasting utilization rates of about 0·5 m-mole/min. were obtained. At higher plasma acetate concentrations, of the order reached after feeding, this increased to 1·8 m-moles/min.

5. Utilization rates were altered by changes in carbohydrate metabolism, e.g. infusion of glucose or insulin.

6. Both [*carboxy*-^{14}C]acetate and [*methyl*-^{14}C]-acetate were used. Taking account of results from both, it would seem that in starved sheep about 6 % of expired carbon dioxide is derived by direct oxidation of acetate. At the higher levels found after feeding, 35 % or more may be so derived.

The advice and encouragement of Sir Rudolph Peters, F.R.S., are gratefully acknowledged. Miss J. Childs, Mr F. Hills and Mr P. C. Wright gave valuable technical assistance.

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Condensed Tannins

8. THE ISOLATION AND DISTRIBUTION OF INTERRELATED HEARTWOOD COMPONENTS OF *SCHINOPSIS* SPP.*

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(*Received* 4 *July* 1960)

Roux & Maihs (1960*a*) showed that the distribution of C_{15} 'flavonoid' compounds of the resorcinol series was confined mainly to certain genera of the Leguminosae and two genera of the Anacardiaceae. Roux (1958, 1959) also demonstrated the association between monomeric flavan-3:4-diols and polymeric tannins of the same structural pattern in both families, and later Clark-Lewis & Roux (1959) found that (+)-7:3′:4′-trihydroxyflavan-3:4-diol of wattle heartwood (Keppler, 1957) *Acacia mearnsii* (syn. *A. mollissima*) and (−)-7:3′:4′-trihydroxyflavan-3:4-diol of quebracho heartwood *Schinopsis quebracho-colorado* (syn. *S. lorentzii*) (Roux, 1958; Freudenberg & Weinges, 1958) were enantiomorphs. Similar distribution of enantiomorphs in the Leguminosae and Anacardiaceae are to be found in the (+)-fustin of *A. mearnsii* (Roux & Paulus, 1960) and (−)-fustin of *Cotinus coggygria* (Freudenberg & Weinges, 1959; Weinges, 1959).

Recent demonstration of the association of the stereochemically interrelated group of flavonoids (+)-7:3′:4′-trihydroxyflavan-3:4-diol, (+)-fustin and (−)-fisetinidol in *A. mearnsii* (Roux & Paulus, 1960, 1961), *Acacia decurrens*, *Acacia dealbata* and *Acacia pycnantha* (Roux, Maihs & Paulus, 1961) (Leguminosae) heartwoods now lends interest to a detailed examination of *S. quebracho-colorado* and *Schinopsis balansae* (Anacardiaceae) heartwoods for the presence of these and possibly other related substances of opposite rotation.

* Part 7: Roux & Paulus (1961).

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points were on molecular mixtures of substances (Roux & Maihs, 1960*a*). Analyses of C, H and methoxyl are by Weiler and Strauss, Oxford. Infrared-absorption curves are by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. A 2 dm. tube was used for optical rotations.

Isolation of fustin, protocatechuic acid, 2-benzyl-4′-methoxy-2:6:3′-trihydroxycoumaran-3-one, gallic acid and catechin from commercial quebracho extract

Powdered 'ordinary' (i.e. unsulphited) quebracho extract (250 g.) prepared commercially from both *S. balansae* (mainly) and *S. quebracho-colorado* (Howes, 1953) was dissolved in 600 ml. of boiling methanol; the mixture was cooled and 800 ml. of chloroform was slowly added with shaking. The precipitate formed was sucked off and the filtrate evaporated to dryness under vacuum in a rotary evaporator (144·5 g.). The powdered solids were dispersed in 400 ml. of commercial boiling ethyl acetate, and ethanol (75 ml.) was added to effect complete solution. After cooling, 200 ml. of chloroform was added as before. The filtrate gave 40 g. of solids after removal of the precipitate.

Solids (80 g.) obtained by the above method were separated in a Craig machine as described by Roux & Paulus

(1960, 1961) and after 160 transfers the tubes were examined by paper chromatography on Whatman no. 1 paper.

(±)-*Fustin*. Contents of tubes 131–146 were run off separately, the organic phase was separated, and the aqueous phase extracted with ethyl acetate. The combined organic phases were evaporated to small volume (50 ml.) and streaked on sheets (5 ml./sheet) of 22½ in. × 18½ in. Whatman no. 3 chromatographic paper. The chromatograms were developed by upward migration in 2% acetic acid for 12 hr. Fustin (R_F, 0·42) was located with toluene-*p*-sulphonic acid spray reagent (Roux, 1957*b*) as a yellow fluorescent band under ultraviolet light. Bands were cut out and eluted with 70% ethanol. A white–pink sludge, containing some crystalline material, separated from the concentrate of the eluates when kept at 0° for 3 days. Brief but rapid heating of the solution caused the sludge to redissolve, leaving fustin crystals. These were sucked off without delay. Recrystallization (charcoal) afforded 80–100 mg. of white crystals m.p. 215–217°. Mixed m.p. with (±)-fustin from *Rhus glabra* (Roux & Paulus, 1960) showed no depression; $[\alpha]_D^{23}$ −9·6±0·6° in acetone–water (1:1, v/v) (*c*, 0·8). Infrared-absorption curves of these fustins were superimposable over the range 2·5–15 μ. Fustin (70 mg.) from commercial quebracho extract was acetylated as for (+)-fustin (Roux & Paulus, 1960), giving 80 mg. of tetra-acetylfustin, m.p. 149°, $[\alpha]_D^{23}$ −7·2±0·2° in tetrachloroethane (*c*, 1·0). Mixed m.p. with tetra-acetyl-(±)-fustin showed no depression.

Compared with the pure optical isomers (−)-fustin, $[\alpha]_D$ −26° (Weinges, 1959), and (+)-fustin, $[\alpha]_D$ +28·3°, and their tetra-acetyl derivatives, $[\alpha]_D$ −25·2° and +24·4° respectively (Roux & Paulus, 1960), the rotation of (±)-fustin and its acetate from quebracho apparently consists of a 2:1 proportion of (−)- and (+)-enantiomers.

Protocatechuic acid. Substance *A* (R_F, 0·50), running in approximately the same position as fisetinidol relative to fustin (Roux & Paulus, 1961), was obtained (30 mg.) from tubes 116–130 by the procedure outlined above. After recrystallization its m.p. (197–199°) and crystal shape (needles) differed from those of fisetinidol (m.p. 211–214°, rhombic), and a mixed m.p. with authentic protocatechuic acid showed no depression. Infrared-absorption curves of substance *A* and protocatechuic acid were identical over the range 2·5–15 μ. Substance *A* and protocatechuic acid gave identical colorations with ferric alum (olive green), diazotized *p*-nitroaniline (Swain, 1953) (canary yellow) and when oversprayed with sodium carbonate (lilac), and substance *A* was also acidic.

2-Benzyl-4′-methoxy-2:6:3′-trihydroxycoumaran-3-one. Component *B* was eluted from bands from the same sheets from which protocatechuic acid and (±)-fustin were obtained. The bands were located with ammoniacal silver nitrate spray and eluted with 70% ethanol as before. White crystals (200 mg.) separated easily from the eluates after concentration, m.p. 194°. Recrystallization from water (charcoal) increased the m.p. to 196°; $[\alpha]_D^{23}$ −1·2±0·2° in acetone–water (1:1, v/v) (*c*, 1·0) (Found: C, 64·7; H, 5·2; −OMe, 8·9. Calc. for $C_{16}H_{14}O_6$: C, 63·6; H, 4·7; −OMe, 10·3%). These values agree fairly closely with those obtained by King (1957) for a compound running to approximately the same position as component *B* (cf. King & White, 1957*a*), and to which he has assigned the structure 2-benzyl-4′-methoxy-2:6:3′-trihydroxycoumaran-3-one.

The infrared-absorption spectrum (KBr) of component *B* has strong absorption bands at 3250 cm.$^{-1}$ (hydroxyl), at 1690 cm.$^{-1}$ (non-chelated aryl ketone), at 1615, 1590 and 1508 cm.$^{-1}$ (C = C aromatic), 1270 cm.$^{-1}$ (C—O aromatic), 1120 cm.$^{-1}$ (aryl—O—C—) and 1038 cm.$^{-1}$ (OMe) (see Stamm, Schmid & Buchi, 1958), which appear to confirm the structure proposed by King (1957).

(+)-*Catechin*. The solids (8 g.) from tubes 66–71 were separated by chromatography on 20 sheets of Whatman no. 3 paper (400 mg./sheet) with 2% (v/v) acetic acid as irrigant. Compound *C* was located by spraying a test strip with bisdiazotized benzidine (Lindstedt, 1950). Two-dimensional chromatography showed that gallic acid accompanies compound *C* in the eluates. The solids (1·6 g.) were separated on eight sheets of Whatman no. 3 paper, previously washed in water, by using butan-1-ol–acetic acid–water (6:1:2, by vol.) (Nordström & Swain, 1953) as irrigant. Bands corresponding to catechin and gallic acid were eluted. The catechin (0·1 g.), although apparently pure, did not crystallize but gave the identical colour reactions and ran to the same position as (+)-catechin on a two-way paper chromatogram with butan-2-ol saturated with water, followed by 2% acetic acid, which permits the separation of all stereoisomers of catechin.

(−)-7:3′:4′-Trihydroxyflavan-3:4-diol, which is present in the same tubes as (+)-catechin (see below) when peripheral heartwood components are subjected to Craig separations, was entirely absent from the commercial extract.

Gallic acid. Gallic acid (200 mg.), m.p. and mixed m.p. with authentic specimen 253°, was obtained from the (+)-catechin fraction (Craig tubes 66–81), although most of the gallic acid is distributed in tubes 81–101.

(±)-*Fustin from the heartwood of* Schinopsis quebracho-colorado (*syn.* Schinopsis lorentzii)

Wood from the outer perimeter of the heartwood of a 42-year-old specimen was removed by drilling with a ⅞ in. drill. The drillings (500 g.) were extracted three times with methanol in the cold (24 hr./extraction), and the combined extracts were evaporated to dryness in a rotary evaporator. The solids (120 g.) were easily soluble in the lower phase (400 ml.) of water–butan-1-ol–light petroleum (b.p. 70–90°) (5:3:2). The solution was introduced into the first eight tubes of a 50 ml. underphase Craig machine, and the mixture separated by countercurrent partition separation as described by Roux & Paulus (1960, 1961).

Fisetin (146–160), fustin (116–141), 2-benzylcoumaranones (King, 1957; King & White, 1957*a*, *b*) (96–121), gallic acid (55–101), (−)-7:3′:4′-trihydroxyflavan-3:4-diol (40–80) and (+)-catechin (50–80) were present in the tube numbers indicated. (+)-Catechin, (−)-7:3′:4′-trihydroxyflavan-3:4-diol and gallic acid are the main polyphenolic components and have been isolated from this wood (Roux & Evelyn, 1960). Fustin (200 mg.) was separated from tubes 116–141 by the methods described above, but tannins did not interfere with their crystallization; m.p. 220°. $[\alpha]_D^{23}$ −3·8±0·6° in acetone–water (1:1, v/v) (*c*, 0·8). Tetra-acetyl derivative, m.p. 150–151°. $[\alpha]_D^{23}$ +0·9±0·3° in tetrachloroethane (*c*, 1·1). Mixed m.p. with tetra-acetyl-(±)-fustin showed no depression.

Table 1. *Radial and vertical distribution of the heartwood components of* Schinopsis quebracho-colorado (42 *years old*)

Benzylcoumaranones separate in the solvent systems used, but could not be estimated accurately as 2-benzyl-2:6:3′:4′-tetrahydroxycoumaran-3-one was not isolated, and the isolated 4′-methoxyl derivative showed slow reducing action. Their relative concentrations were therefore indicated by + and × respectively. In sample 2 (ground level) the 4′-methoxyl derivative appears to be present over the range 1–3%.

Average height above ground of cross-section (in.)	Sampling position	(−)-7:3′:4′-Trihydroxy-flavan-3:4-diol (%)	(+)-Catechin (%)	(±)-Fustin (%)	Fisetin (%)	Benzyl-coumaranones	Gallic acid (%)
		Sample 1 (Roux & Evelyn, 1960)					
206·5	1	6·0	3·7	0·0	0·0	–	1·5
104	02	9·5	3·8	1·4	0·0	–	1·7
	1	4·5	4·3	1·6	0·0	+	1·3
	2	8·0	3·3	1·0	0·1	+	1·2
37	04	5·7	3·6	0·0	0·0	–	1·6
	03	7·3	2·5	1·0	1·2	+	1·7
	02	5·5	1·6	0·8	1·3	+ +	1·4
	1	2·6	1·2	1·1	1·1	+ +	0·7
	2	4·3	1·6	0·6	1·0	+ + × ×	1·5
	3	5·5	3·0	0·8	0·6	+ + × ×	1·3
	4	4·6	3·6	0·2	0·0	–	2·5
8	05	5·7	2·6	1·1	0·4	+ ×	2·2
	04	2·3	1·7	1·3	0·8	+ + ×	1·6
	03	1·1	1·7	0·8	1·1	– ×	2·2
	02	2·6	1·4	1·3	1·8	+ + ×	1·9
	1	4·2	1·9	1·1	2·0	+ + ×	1·9
	2	1·3	2·2	1·4	1·3	+ ×	2·2
	3	6·4	3·6	1·2	0·4	–	3·3
		Sample 2 (Roux & Evelyn, 1958)					
Ground level	05	3·2	1·9	0·3	2·5	+ + +	2·8
	04	3·0	1·1	0·3	3·0	+ + (×)	2·5
	03	3·0	1·3	1·4	3·0	+ × × ×	1·9
	02	0·8	0·7	1·5	2·2	(+) × × ×	1·3
	1	0·2	0·6	1·7	2·2	(+) × × ×	1·5
	2	1·2	0·6	1·2	2·3	– × ×	1·7
	3	2·7	1·3	1·2	2·0	(+) × ×	2·0
	4	4·2	1·8	1·4	3·0	+ + × ×	2·5
	5	1·9	1·4	0·5	1·6	(+)	2·8

(−)-7:3′:4′-Tetrahydroxyflavan-3:4-diol (900 mg.) was isolated from tubes (40–80) by the same methods as those employed for the (±)-fustin.

Absence of (+)-*fisetinidol.* (−)-7:3′:4′-Tetrahydroxyflavan-3:4-diol (500 mg.) was hydrogenated as described by Weinges (1958) to give 300 mg. of (+)-fisetinidol, m.p. 211–214°. Chromatographic comparisons showed that (+)-fisetinidol was completely absent from those Craig tubes (110–140) in which its presence was expected.

(±)-*Fustin from the heartwood of* Schinopsis balansae

Three small specimens of the heartwood from different regions of the Argentine were combined (500 g.), and the methanol extractives of the drillings and powdered wood (130 g.) were separated in the Craig machine. Fustin, m.p. 220° (100 mg.), was isolated as before, and converted into the tetra-acetyl derivative m.p. 146–147°; $[\alpha]_D^{23}$ −6·1±0·4° in tetrachloroethane (*c*, 0·8). Compared with the specific rotation of the pure tetra-acetyl-(−)-fustin (24·4°), this value shows that the racemate contains approx. 62·5% of (−)-fustin. (+)-Fisetinidol was also absent in the heartwood components of *S. balansae*. Traces of protocatechuic acid were present.

Radial and vertical distribution of (−)-7:3′:4′-*trihydroxyflavan*-3:4-*diol,* (+)-*catechin, gallic acid, fustin, fisetin and* 2-*benzylcoumaranones in* Schinopsis quebracho-colorado

Radial samples of cross-sections of a 42-year-old specimen of *S. quebracho-colorado*, obtained by drilling, have been examined for average molecular weight of tannins, total leuco-fisetinidin content and tannin/non-tannin ratio (Roux & Evelyn, 1958) and also vertically at different heights (Roux & Evelyn, 1960). By the methods of Roux & Maihs (1960*a*) the radial and vertical distributions of the above compounds were estimated by chromatographic and densitometric methods with the identical specimens

examined previously (Roux & Evelyn, 1958, 1960). Ammoniacal silver nitrate spray reagent was used for (−)-7:3′4′-trihydroxyflavan-3:4-diol and bisdiazotized benzidine (Lindstedt, 1950) for (+)-catechin. Gallic acid was estimated with bisdiazotized benzidine, but, owing to overlap of components, aq. 3% ferric alum reagent, freshly made, was used for visual confirmation of the concentration. Fisetin, fustin and the 2-benzylcoumaranones were estimated from sheets where the ammoniacal silver nitrate spray had been used. In some samples, where slight overlap of other components occurred, visual estimation of fisetin and fustin provided a check on the accuracy of the densitometric method. Precise details of the spray reagents and methods are described by Roux & Maihs (1960*b*). The distribution of components is shown in Table 1.

DISCUSSION

King & White (1957*a*, *b*) have already drawn attention to a biogenetic metabolic relation between the structurally related compounds 2-benzyl-2:6:3′:4′-tetrahydroxycoumaran-3-one, its 4′-methyl ether, fisetin, 4′-methoxyfisetin, fustin and leuco-fisetinidin, all of which occur in quebracho heartwoods. The benzylcoumaranones (King, 1957) and the fisetins (Perkin & Gunnell, 1896; Kirby & White, 1955) and (−)-7:3′:4′-trihydroxyflavan-3:4-diol (Roux, 1958; Freudenberg & Weinges, 1958; Roux & Evelyn, 1958) have been isolated and chromatographic evidence has been given for the presence of fustin (King & White, 1957*a*).

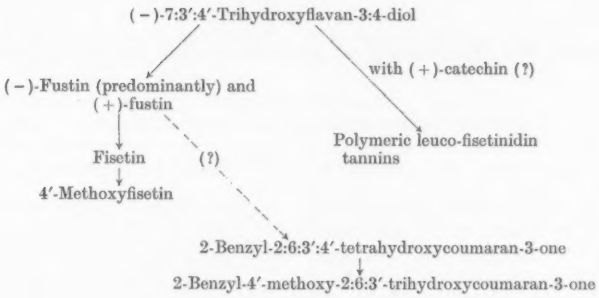
Fustin has now been isolated in low yield (0·02%) from commercial quebracho extract and from the heartwoods of *S. balansae* and *S. quebracho-colorado*. The high melting points of these fustin acetates (146–151°) compared with the pure optical isomers (116–118°) showed that fustin was present as a racemate in each instance (Roux & Paulus, 1960). A predominance (66%) of (−)-fustin was, however, present in the commercial extract and in samples of *S. balansae* examined. In view of the association of related 2:3-dihydroflavonols and flavan-3:4-diols of the same rotation in a number of woods (Weinges, 1958; Freudenberg & Weinges, 1959; Weinges, 1959; Roux & Paulus, 1960) the association of (−)-fustin and (−)-7:3′:4′-trihydroxyflavan-3:4-diol in the heartwoods of *Schinopsis* spp. was expected. This appears at least partly valid for the commercial extract derived mainly from the heartwood of *S. balansae* (Howes, 1953).

(+)-Fisetinidol, a catechin which is related chemically and stereochemically to (−)-7:3′:4′-trihydroxyflavan-3:4-diol (Weinges, 1958) was absent from *S. balansae*, *S. quebracho-colorado* and the commercial extract. Protocatechuic acid was isolated from the commercial extract and is also present in traces in *S. balansae* heartwood.

Of the benzylcoumaranones (King & White, 1957*a*; King, 1957) the 4′-methoxyl derivative of 2:6:3′:4′-tetrahydroxycoumaran-3-one apparently predominates in the commercial extract and may readily be separated by Craig countercurrent separation followed by adsorptive separation on cellulose. The optical inactivity (King, 1957) of the substance was confirmed, and an interpretation of its infrared absorption curve apparently supports the structure assigned by King (1957).

Estimation of the radial and vertical distribution of (−)-7:3′:4′-trihydroxyflavan-3:4-diol and (+)-catechin in *S. quebracho-colorado* confirms the visual observation (King & White, 1957*a*; Roux, 1958; Roux & Evelyn, 1958) of their decrease with increasing age of the heartwood, and provides an accurate assessment of their distribution in the heartwood for the first time. Changes which occur in the concentration of these substances may now be linked directly with the changes in tannin/non-tannin ratio, average molecular weight and total leuco-fisetinidin content observed for the same specimens (Roux & Evelyn, 1958, 1960). (−)-7:3′:4′-Trihydroxyflavan-3:4-diol and (+)-catechin are present in high concentration in the first heartwood to be laid down in the growing tree (height 206·5 in., Table 1) and in the peripheral heartwood (heights 37, 8 and 2 in., Table 1) in the mature portion of the tree. These compounds increase in concentration slightly further inwards (at 37, 8 and 2 in. levels) in the mature heartwood and where the young heartwood widens (104 in. level), to reach a maximum, and then decline in concentration toward the centre of the heartwood (104, 37 and 2 in. levels). The even gradation of (−)-7:3′:4′-trihydroxyflavan-3:4-diol is sometimes upset (8 in. level), possibly owing to some growth irregularity. Gallic acid concentration also declines slightly towards the centre of the heartwood. In trees of advanced age (over 100 years old) the peripheral heartwood containing the (−)-7:3′:4′-trihydroxyflavan-3:4-diol and (+)-catechin is very narrow, and the central heartwood from which the commercial extract is derived (Howes, 1953) contains no (−)-7:3′:4′-trihydroxyflavan-3:4-diol, a trace of (+)-catechin, but relatively high concentrations of gallic acid.

The progressive decrease of (−)-7:3′:4′-trihydroxyflavan-3:4-diol with age of the heartwood appears to be associated with increases in the proportions of (±)-fustin, fisetin and related benzylcoumaranones (Table 1). This, together with the predominance of (−)-fustin in *S. balansae*, suggests that in addition to the formation of leuco-fisetinidin tannins (Roux, 1957*a*, *b*) interconversions may also occur according to Scheme 1. The origin of the 2-benzyl-2-hydroxycoumarones is not obvious, but they have been formed by the action of alkali on dihydroflavonols (Gripenberg, 1957). The radial decline of (+)-catechin possibly indicates that this substance



Scheme 1

may also be involved in tannin formation as suggested by King & White (1957*a*, *b*).

These radial changes in the heartwood of *Schinopsis* spp. are accompanied by a parallel increase in molecular weight and tannin/non-tannin ratio (Roux & Evelyn, 1958, 1960), as well as by an apparent pattern of interconversion and condensation of a parent flavan-3:4-diol similar in some respects to that visualized for the heartwood of *A. mearnsii* (Roux & Paulus, 1961).

SUMMARY

1. (±)-Fustin has been isolated from the heartwoods of *Schinopsis balansae*, *Schinopsis quebracho-colorado* and from commercial quebracho extract. (−)-Fustin predominates (66%) in the extract of *S. balansae* and in the commercial extract.

2. Other known and some related components have also been isolated by means of Craig countercurrent separations followed by adsorption separation on cellulose.

3. The radial and vertical distribution of (−)-7:3′:4′-trihydroxyflavan-3:4-diol, (+)-catechin, gallic acid, (±)-fustin, fisetin and 2-benzyl-2-hydroxycoumaran-3-ones in the heartwood of *S. quebracho-colorado* has been estimated.

4. The possible role these compounds play in the biogenesis of tannins and the interconversions of the compounds related to (−)-7:3′:4′-trihydroxyflavan-3:4-diol are discussed.

This work is financed by the annual grant of the South African Wattle Growers' Union to the Leather Industries Research Institute.

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Biochem. J. (1961) **78**, 790

A New Reagent for the Assay of Indole in the Tryptophanase Reaction

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(*Received* 20 *July* 1960)

Harley-Mason & Archer (1958) introduced *p*-dimethylaminocinnamaldehyde as a spray reagent for the detection of indole derivatives on paper chromatograms. This vinyl analogue of *p*-dimethylaminobenzaldehyde gave intensely coloured condensation products with a tenfold increase in sensitivity. The following describes the use of *p*-dimethylaminocinnamaldehyde as a substitute for the benzaldehyde derivative, in a modification of the indole-assay procedure developed by Happold & Hoyle (1934) for the measurement of tryptophanase activity.

EXPERIMENTAL

Indole-extraction procedure. The procedure for extraction of indole into light petroleum, as described originally by Happold & Hoyle (1934), has since been modified in these laboratories. Details of the modified procedure used in the present work are as follows.

Aqueous solutions containing indole are made to a total of 20 ml. Most of the indole is then extracted into 40 ml. of light petroleum (b.p. 40–60°) by vigorous shaking in 250 ml. conical separating funnels, the aqueous layers being discarded.

At 20·5° the distribution coefficient of indole between light petroleum and water is 5·01 (Scott, 1960), and at equilibrium 91% of the indole is extracted into the organic solvent layer by a single extraction. Careful standardization of the volumes of aqueous and organic phases during extraction is essential; a 25% deviation from the volume of aqueous phases gives rise to a 2·2% deviation in the indole content of the organic phase. During extraction from aqueous solutions containing denatured protein, slow phase separation can be avoided by filtration through a plug of glass wool, which is then washed (Scott, 1960).

Production and measurement of colours. To indole contained in 40 ml. of light petroleum is added 10 ml. of Ehrlich reagent or *p*-dimethylaminocinnamaldehyde reagent. After vigorous shaking, the coloured aqueous layer is drained into tubes and centrifuged. The coloured solutions are transferred to spectrophotometer tubes by Pasteur pipette and the colour densities read in a Unicam SP. 1400 diffraction-grating spectrophotometer, usually with pure reagent as blank. Colours are read 15–20 min. after addition of Ehrlich reagent at 560 mμ, and after exactly 30 min. at 640 mμ with *p*-dimethylaminocinnamaldehyde reagent. Readings are referred to a calibration curve constructed by extracting known amounts of indole in aqueous solution by the above-described procedure.

Ehrlich reagent is prepared by dissolving 16 g. of *p*-dimethylaminobenzaldehyde in 1250 ml. of ethanol and then adding 320 ml. of concentrated hydrochloric acid. The reagent is 67 mM with respect to *p*-dimethylaminobenzaldehyde and approx. 2N with respect to hydrochloric acid.

p-Dimethylaminocinnamaldehyde reagent was finally prepared by dissolving 2·34 g. in 780 ml. of ethanol, adding 100 ml. of concentrated hydrochloric acid and making up to 1000 ml. with water. This reagent is 13·4 mM with respect to *p*-dimethylaminocinnamaldehyde, and approximately N with respect to acid.

Standard solutions of indole were prepared by dissolving freshly sublimed samples in ethanol to give a 1% (w/v) solution. The ethanolic solution was then diluted, usually to 10 μg. of indole/ml., with water.

RESULTS AND DISCUSSION

Substitution of an equimolar amount of *p*-dimethylaminocinnamaldehyde in the preparation of Ehrlich reagent yielded a highly coloured solution which absorbed strongly between 550 and 650 mμ. To facilitate zero adjustment for the reagent blank, the *p*-dimethylaminocinnamaldehyde concentration was decreased to one-tenth of this concentration. With 10 ml. of this diluted reagent, approx. 10 μg. of indole was extracted from light petroleum by the usual procedure. The absorption spectrum of the dark-green colour thus produced showed a strong absorption peak at 646 mμ [absorption maximum was observed to be 625 mμ with a Unicam SP. 500 spectrophotometer (Scott, 1960)], although the absorption of this peak rapidly decreased with time. Colour fading was not dependent on light.

In an attempt to measure the rates of colour formation and decay 9 ml. of *p*-dimethylaminocinnamaldehyde reagent was added to 1 ml. of aqueous indole solution (10 μg. of indole) contained in a spectrophotometer tube. Absorption at 640 mμ reached a maximum after 7 min., and the colour was stable over the next 15 min. The improved colour stability was concluded to be a result of decreased concentration of acid during colour development. The absorption spectrum of the colour, plotted during the period of colour stability, is shown in Fig. 1.

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Effect of concentration of hydrochloric acid on colour development and stability. A series of reagent mixtures were prepared, with a standard solution of *p*-dimethylaminocinnamaldehyde in ethanol, such that, after dilution with various proportions of concentrated hydrochloric acid and water, the overall concentration of reagent was 6·7 mM. The development and decay of colours obtained by extracting approx. 20 μg. of indole from light petroleum with the series of reagents, by the usual procedure, are shown in Fig. 2. The conclusions drawn from these and other results were that as the concentration of hydrochloric acid in the reagent increases, the rate of development of maximum colour intensity increases but the final colour intensity and the time period over which the colour is stable decrease. In the present work, it was decided to compromise between a rapid development of colour, high colour intensity and stability. The reagent used in all further work was approx. N with respect to hydrochloric acid.

Effect of concentration of p-*dimethylaminocinnamaldehyde on colour density.* This factor was investigated with 5 μg. of indole, as it was amounts of indole of this order that were of interest. The maximum colour density was measured at 640 mμ with various concentrations of *p*-dimethylaminocinnamaldehyde after periods of exactly 30 min. colour development (Table 1). From these results it appeared that, under the conditions used, a suitable reagent concentration was 13·4 mM with respect to *p*-dimethylaminocinnamaldehyde.

Sensitivity of p-*dimethylaminocinnamaldehyde and Ehrlich reagents.* A calibration curve was constructed by light-petroleum extraction of between

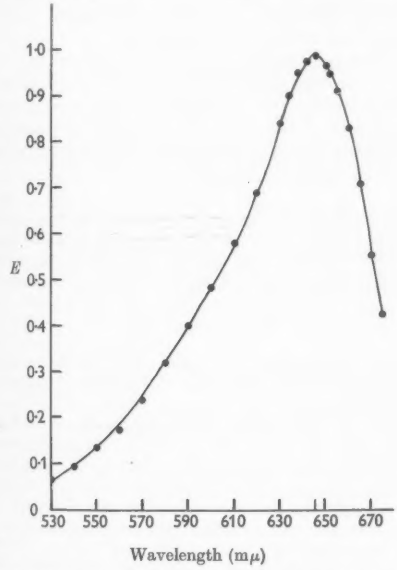

Fig. 1. Absorption spectrum of *p*-dimethylaminocinnamaldehyde colour with indole. To 9 ml. of *p*-dimethylaminocinnamaldehyde reagent, 6·7 mM, and 2N with respect to acid, was added 10 μg. of indole in 1 ml. of aqueous solution. The spectrum was plotted between 7 and 15 min. after mixing, with water replacing indole solution in the blank. The absorption maximum was observed at 646 mμ.

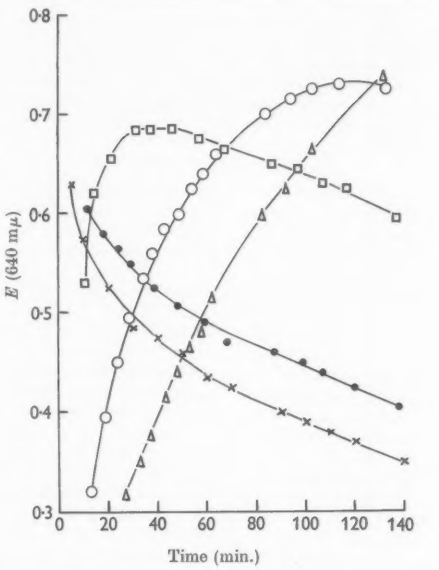

Fig. 2. Effect of acid normality on colour development and stability. Aqueous indole solutions, 20 μg. in 20 ml., were extracted with 20 ml. of light petroleum. Colours were developed with 10 ml. of *p*-dimethylaminocinnamaldehyde reagent, 6·7 mM, at various acid normalities: 0·3N, △; 0·5N, ○; 1·0N, □; 1·7N, ●; 2·3N, ×. Colour development was followed at 640 mμ.

Table 1. *Effect of* p-*dimethylaminocinnamaldehyde on colour density*

Aqueous solutions of indole, 5 μg. in 20 ml., were extracted with 40 ml. of light petroleum, and colours were developed with *p*-dimethylaminocinnamaldehyde reagent, N with respect to acid, as described in the text.

Final concn. of reagent (mM)	*E* at 640 mμ after 30 min.
6·7	0·193
13·4	0·264
20·1	0·266

1 and 10 μg. of indole from 20 ml. of aqueous solution by the standard procedure. Colour development and measurement, with *p*-dimethylaminocinnamaldehyde reagent, were carried out as described above. The results are shown in Fig. 3. Included in Fig. 3 is part of a linear calibration curve obtained with Ehrlich reagent. For amounts of indole below 10 μg. of the latter reagent gives poorly reproducible results, whereas *p*-dimethylaminocinnamaldehyde reagent has been found to give approx. $\pm 5\%$ accuracy for amounts of indole above 1 μg. If *p*-dimethylaminocinnamaldehyde reagent containing hydrochloric acid at an overall normality of less than 1 is used, and with longer standard development times, the method is more than 2·2 times as sensitive as the method with Ehrlich reagent.

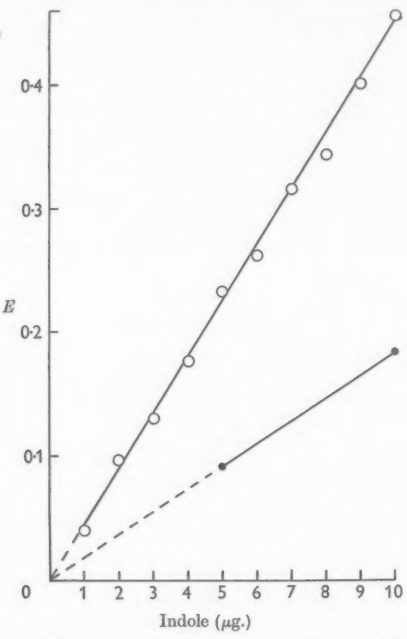

Fig. 3. Indole calibration curves with *p*-dimethylaminocinnamaldehyde reagent and Ehrlich reagent. The indole-extraction procedure, also the production and measurement of colours, were carried out as described in the Experimental section. Extinction values: with *p*-dimethylaminobenzaldehyde (Ehrlich) reagent, ●; with *p*-dimethylaminocinnamaldehyde reagent, ○.

p-Dimethylaminocinnamaldehyde reagent has been used in these laboratories for the assay of tryptophanase activity during purification studies. The increased sensitivity of the reagent was found to be valuable when limited amounts of fractions from *Escherichia coli* extracts were available. The reagent has also been used (T. A. Scott, unpublished work) for the indirect assay of small amounts of tryptophan by initial enzymic breakdown to indole.

SUMMARY

1. Details are presented of a modified procedure for the extraction of indole into light petroleum from aqueous tryptophanase reaction systems.

2. The use of *p*-dimethylaminocinnamaldehyde as a reagent for indole is described, and factors affecting the rate of colour formation, colour density and stability are described.

3. The quantitative use of this reagent is compared with that of Ehrlich reagent. The sensitivity increase under the conditions selected is greater than 2·2, and the reagent is capable of assaying amounts of indole greater than 1 μg. contained in 20 ml. of aqueous solution.

This work has been carried out with the support of the Medical Research Council. The author is grateful for a Medical Research Council training scholarship, also for the helpful encouragement of Professor Frank C. Happold.

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The Prosthetic Group of Cytochrome Oxidase

1. PURIFICATION AS PORPHYRIN *a* AND CONVERSION INTO HAEMIN *a*

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(*Received* 4 *July* 1960)

Earlier publications from this Laboratory (Lemberg, Bloomfield, Caiger & Lockwood, 1955; Lemberg & Stewart, 1955) have described the preparation of porphyrin *a* from ox heart and the conversion of this porphyrin into haemin *a*.

Further studies on the structure of haematin *a* (Clezy & Barrett, 1961), its identification as the prosthetic group of cytochromes *a*, a_3 and a_1 and studies on the spectra of its various derivatives have required further improvement in the purification of porphyrin *a* and its preparation on a larger scale than hitherto. A preparation of porphyrin *a* of higher spectroscopic purity than previously described and a method for its conversion into haemin *a* without alteration are given in the present paper.

EXPERIMENTAL

Methods

Hydrochloric acid solutions. Throughout this paper the concentrations of aqueous HCl solutions are given in % (w/v) to conform with the convention in this field.

Ether. 'Technical' diethyl ether which did not contain added reducing agents such as quinol was used. Except where otherwise stated ethereal solutions were evaporated under vacuum in a water bath at 50°.

Acetone. Commercial acetone was used for extraction of lipids from mince and for acid–acetone extractions of haemins; for other uses including chromatography AnalaR-grade acetone was used.

Acetic acid. That designated 'specially treated' in the text was an AnalaR-grade reagent refluxed for 1 hr. after the addition of a small amount of $FeSO_4$ and then distilled; otherwise the AnalaR-grade reagent was used.

Cellulose powder. Whatman cellulose powder 'Standard Grade, Ashless Powder Chemically Prepared' (Balston Ltd., England) was used. Tubes $1\frac{1}{2}$ in. × 48 in. or 1 in. × 48 in. were packed as indicated in the text with dry cellulose, the lower end being tapped frequently on a solid surface during packing to consolidate the powder. This procedure was more satisfactory than packing the cellulose as slurry in the solvent.

Light-absorption measurements. These were made with a Hilger 'Uvispek' spectrophotometer with the glass prism. The wavelength setting was adjusted by using the hydrogen lines at 486·1 and 656·3 mμ.

Paper chromatography of porphyrin a. Two solvent systems were used: 1. Propanol–kerosene (commercial grade, Shell Co. Ltd.) (1:5, v/v) on Whatman no. 1 paper, the solvent front being allowed to ascend 10 cm. in a kerosene atmosphere. 2. Acetone–light petroleum (b.p. 60–80°) (1:3·5, v/v) on Whatman no. 1 paper strips 1·0–1·5 cm. wide suspended from the stopper in test tubes containing the solvent mixture. The solvent front was allowed to ascend 4–6 cm. Since porphyrin *a* dried on paper has a poor fluorescence the chromatograms were viewed under ultraviolet–blue light after being sprayed with kerosene.

Purification of porphyrin a

Preparation of defatted mince. About 18 kg. of lean ox-heart mince was washed for 30 min. in running tap water, soaked in 3% (w/v) NaCl for a further 30 min. and again washed with running tap water for 30 min. The strained mince was squeezed, with double layers of muslin, to remove as much water as possible and successively extracted for 1 hr. each time with 20 l. of acetone–water (4:1, v/v) (sp.gr. of mixture, 0·875), and 12·5 l. of acetone–ether (1:1), and kept overnight in 12·5 l. of acetone–water (4:1).

Extraction of haemins. After removal of excess of solvent, the mince was extracted once for 15 min. and twice for 30 min. with separate mixtures each containing 20 l. of acetone, 4 l. of water and 1 l. of conc. HCl. The extracted haemins were transferred to ether without delay by using 4% HCl to wash out the acetone. The ether from the combined solutions was evaporated under reduced pressure in six or seven 2 l. flasks immersed in hot water. A small amount of residual water could usually be poured off, leaving a fatty residue containing the haemins.

The haemins should not be kept in acetone–HCl any longer than necessary since the formyl group of formylhaemins slowly condenses with acetone under these conditions (Clezy, 1959).

Precipitation of protohaemin and cryptohaemin a. The fatty haemin residue was shaken by hand for a few minutes at room temperature with 2 l. of ether and filtered through a no. 541 Eaton–Dikeman fluted paper (Eaton–Dikeman Co., Mount Holly Springs, Pa., U.S.A.) to remove undissolved protohaemin and most of the cryptohaemin *a*. The residue on the paper was washed with 1 l. of ether, and the combined filtrates were evaporated to 300 ml. under reduced pressure. At this stage the band of haemin *a* at 650–660 mμ was considerably stronger than the protohaemin band at 630–640 mμ when viewed in the hand spectroscope.

Acetone precipitation of lipids. The solution of haemins was dried for 15 min. over anhydrous Na_2SO_4 and filtered into 1200 ml. of acetone. The residue was washed with 300 ml. of acetone, and the filtrates were combined and made up to 1·8 l. with acetone. Two drops of a saturated aqueous solution of $MgSO_4$ were added, and the mixture was shaken and kept overnight at −15°. The precipitated

lipids were filtered off at $-15°$, the residue being washed with 200 ml. of acetone at $-15°$.

The haemins from four preparations treated as above and derived from a total of 65·5 kg. of mince were combined and dissolved in ether. The volume was brought to 2 l. by evaporation of the ether *in vacuo*, and the solution was dried over anhydrous Na_2SO_4. The ethereal solution was evaporated to dryness *in vacuo* and dissolved in 1·1 l. of acetone. Two drops of saturated $MgSO_4$ solution were added, and the precipitated lipid was filtered off after being kept overnight at $-15°$. The residue was washed with 750 ml. of acetone at $-15°$. The haemins were transferred to 2 l. of ether and dried over anhydrous Na_2SO_4.

Conversion of haemins into porphyrins. The approximate haemin (protohaemin + haemin *a*) content of the ethereal solution was calculated from the extinction at 550 mμ assuming that a solution in ether of 1 mg./ml. would have an extinction of 10 (specific extinction) at this wavelength. By this method 1280 mg. was found to be present in the ethereal solution.

This solution of haemins was then evaporated to dryness and dissolved in 12 l. of specially treated acetic acid (see above). Portions (500 ml.) were successively treated as follows. After CO_2 had been bubbled through each 500 ml. portion for 10 min., 900 mg. of $FeSO_4,7H_2O$ (14 moles/mole of haemin) dissolved in 10 ml. of conc. HCl were added at room temperature. The stream of CO_2 was continued for 5 min. when the solution, now greenish by reflected light, was poured into a 3 l. separating funnel containing about 1 l. each of ether and water plus sufficient sodium acetate to neutralize the solution to Congo red, and then shaken.

Removal of protoporphyrin and cryptoporphyrin a. The ethereal solution of porphyrins was washed twice with approximately an equal volume of water to remove much of the acetic acid and twice with small volumes of 7% HCl to remove the protoporphyrin and cryptoporphyrin *a* not previously precipitated as their haemins. Acetic acid then remaining in the ethereal solution was largely removed by washing twice with an approximately equal volume of 1% HCl and once with water.

Chromatography on cellulose-powder columns. The ethereal solution now contained lipid impurities, porphyrin *a*, traces of protoporphyrin and some protohaemin. The maxima of bands III and IV of the porphyrin *a* were at 558 and 517 mμ respectively. This solution was shaken gently with dil. aq. $NaHCO_3$ solution until the pH of the aqueous phase was 6·0–6·5. Any persistent emulsions were broken by gentle shaking with conc. $(NH_4)_2SO_4$ solution. The ethereal solution was kept at $-15°$ overnight then filtered at $-15°$ to remove ice crystals, which were washed with a little cold ether. A third of the preparation was chromatographed at a time on six columns (36 in. × 1·5 in.) of cellulose powder, each column bearing 16–20 mg. of porphyrin. The porphyrin *a* was applied to the columns in solution in ether–light petroleum (b.p. 60–80°) (1:1, v/v). This was followed by an equal volume of the same solvent mixture, which moved a yellowish lipid fraction, later rejected, well clear of the porphyrin *a* adsorbed on the top third of the column. The column was then developed with ether or 10% (v/v) acetone in ether, which eluted a broad band of porphyrin. The eluate was collected as six or seven separate fractions. The fractions having a ratio of bands III/IV of 1·90 or higher were combined and rechromatographed. From a second or third chromatography, fractions with a ratio of bands III/IV as high as 2·45 were obtained. The pooled porphyrin *a* finally collected had a ratio of band III (558 mμ)/band IV (518 mμ) of 2·35. The yield at this stage, calculated by assuming a specific extinction of 27 at 558 mμ for pure porphyrin *a* (see below), was 297 mg. (from 65·5 kg. of mince). The specific extinction at 558 mμ of the prepared porphyrin *a* was 8·7.

Fractionation with hydrochloric acid. Ethereal solutions containing about 20 mg. of porphyrin *a* were evaporated to dryness under reduced pressure in a 2 l. flask so that the porphyrin was spread thinly on the walls of the flask. Each flask was then kept under vacuum at 60–80° for 30 min. after which the porphyrin *a* was dissolved in ether (a little acetone was sometimes necessary) and chilled. When all the porphyrin had been so treated it was extracted in the cold room (2°) into cold 17% HCl. Extraction at 2° minimizes the conversion of porphyrin $a\alpha$ into porphyrin $a\beta$ (Lemberg & Stewart, 1955). The extract was washed with 2 vol. of ether and diluted with cold water so that it was 8% with respect to HCl. On thorough shaking with ether, porphyrin $a\alpha$ of increased purity went into the ethereal layer. The final yield was 210 mg. of porphyrin *a*.

Spectral characteristics. In solution in ether the porphyrin *a* had absorption maxima at 647 mμ (band I), 582 mμ (band II), 558 mμ (band III) and 518 mμ (band IV). The specific extinction at 558 mμ was 23. The following ratios of absorption maxima are useful in characterizing the porphyrin: band III/band I, 16·2; band III/band II, 1·92; band III/band IV, 2·36.

The millimolar extinction coefficient at 558 mμ for porphyrin *a* in ether was determined on two preparations similar to that described above by the copper titration method of Oliver & Rawlinson (1951) and found to be 21. This value is slightly higher than that given by Oliver & Rawlinson (1951), owing probably to the increased spectral purity of the present porphyrin *a*. From the molecular weight of 870 found for crystalline haemin *a* by Warburg, Gewitz & Völker (1955) the specific extinction of pure porphyrin *a* in ether at 558 mμ was calculated to be 27. Our product is therefore about 85% pure.

Paper chromatography of porphyrin a. This was done as described above. This technique was used to see whether porphyrin *a* existed in more than one chromatographic form. Several preparations, at different stages in the purification, exhibited two spots of about equal intensity. Both solvent systems gave these two spots. In the acetone–light petroleum system the spots had R_F values of about 0·60 and 0·45 and in propanol–kerosene of 0·28 and 0·18. The most highly purified preparations of porphyrin gave mainly the spot of higher R_F with a barely discernible second spot of the lower R_F. In one preparation the spot of higher R_F greatly predominated throughout the purification. It is difficult to judge whether the two spots observed are

due to different porphyrins or to the effect of contaminating lipids.

Interconversion of formyl porphyrins and haemins

Conversion of haemin a *into porphyrin* a. The method given above follows the principles discussed by Morell & Stewart (1956) for haemins generally. With ordinary AnalaR-grade acetic acid, however, removal of iron from haemin *a* produced variable amounts of porphyrin having its band III at 551–553 mμ and which could be separated from porphyrin *a* (band III, 558 mμ) on the cellulose column. This porphyrin was an alteration product of porphyrin *a*, the reaction being caused by an unidentified oxidant in the acetic acid which was active in the presence of Fe^{2+} ions and high proton concentration. The alteration was prevented by using the 'specially treated' acetic acid described above.

The altered porphyrin *a*, which we refer to below as 'porphyrin *a* acid', no longer contained a carbonyl group. It was largely separated from porphyrin *a* by cellulose chromatography and freed from traces of the formyl porphyrin as follows: 10 mg. of porphyrin *a* acid and 100 mg. of Girard T reagent, dissolved in 250 ml. of 95% ethanol, were heated together under reflux for 30 min. After the solution was cooled, the porphyrin was returned to ether and washed with water until the washings were free from water-solubilized porphyrin. The porphyrin in the ethereal layer was identical in spectral and paper-chromatographic behaviour with the carboxylic acid obtained from porphyrin *a* by the oxidation of the formyl group [see the next paper (Clezy & Barrett, 1961)].

Other formyl haemins, namely monoformyl- and 2-formyl-4-vinylferrodeuteroporphyrin (methyl esters), were also converted into porphyrins by using untreated acetic acid, $FeSO_4,7H_2O$ (14 moles/mole of haemin) and conc. HCl. The resulting porphyrin esters were dissolved in ether and extracted with aq. 1% Na_2CO_3 solution. In each case the porphyrin so extracted was redissolved in ether by first adding dilute acetic acid and washed with water. These porphyrins were identical (spectra and R_F values) with the porphyrincarboxylic acids obtained by the CrO_3–H_2SO_4 oxidation of the corresponding formyl porphyrins (Barrett & Clezy, 1959).

Conversion of porphyrin a *into haemin* a. Porphyrin *a* purified as above was converted into the haemin by heating in specially treated acetic acid (see above) at 80° for 10 min. in the presence of 20μmoles of $FeSO_4,7H_2O$/μmole of porphyrin and a few crystals of NaCl. The concentration of porphyrin in acetic acid was 1 mg./10 ml.; sufficient AnalaR Na_2CO_3 was added to this solution so that only the neutral spectrum could be seen. Care was specially required to exclude copper ions from this reagent mixture. After being heated for 10 min. the mixture was cooled, diluted with ether (freshly washed with dil. aq. $FeSO_4$ to remove peroxides), and the acetic acid largely removed by three or four washes with dil. HCl. Traces of porphyrin *a* were then removed with several washes with small volumes of 20% HCl, and the haemin *a* solution was finally washed with 3% HCl.

The effect of the removal and replacement of iron on the molecule was tested by using porphyrin *a* having ratio of bands III/IV of 2·31. This porphyrin was converted into its haemin and then reconverted into the porphyrin. The ratio of the final porphyrin *a* produced was 2·24 and the band positions had not changed.

The haemin *a* prepared by reintroduction of iron into purified porphyrin *a* had absorption maxima (in ether washed with 3% HCl) at 662–663, 552 and 508 mμ. The following ratios of absorption intensities are useful in characterizing the haemin: $E_{552\,m\mu}/E_{590\,m\mu} = 1{\cdot}97$ (to detect traces of copper complex absorbing at 590 mμ); $E_{663\,m\mu}/E_{640\,m\mu} = 1{\cdot}15$ (to detect traces of protohaemin absorbing at 640 mμ). Since haemin *a*, like other haemins, has an absorption shoulder at 590–620 mμ, traces of the copper complex cannot be detected visually in the hand spectroscope. 'Native' haemin *a* is the term used below for haemin *a* purified without conversion into the porphyrin at any stage. The spectrum of haemin *a* prepared as above was compared with 'native' haemin *a*. The position of absorption bands was the same for the two haemins. $E_{552\,m\mu}/E_{590\,m\mu}$ was 1·93 for 'native' haemin *a* but $E_{663\,m\mu}/E_{640\,m\mu}$ was 1·06, indicating some contamination with protohaemin.

'Native' haemin *a* was prepared with very mild procedures to prevent any alteration which might affect its spectrum. The preparation was the same as for the porphyrin *a* preparation up to the stage of the second defatting. In solution in ether the haemin mixture was then chromatographed on cellulose-powder columns. A yellow lipid fraction preceded the haemins down the column and was discarded; some protohaemin, which is adsorbed more strongly to the cellulose, was retained on the columns. After elution of the haemin *a* with ether or ether plus acetone, the fraction containing the haemin *a* was evaporated under reduced pressure to an oily residue and partitioned between aq. acetone–HCl and acetone–light petroleum as described by Kiese & Kurz (1954). After passage three times through this countercurrent system there seemed to be no further separation of protohaemin from haemin *a* as judged from the relative intensities at 558 and 587 mμ of the haemochrome derivatives.

Quantitative measurements of protohaemochrome and haemochrome *a* with the formula

quoted by Lemberg & Benson (1959) showed a protohaemin contamination of the haemin *a* preparation of from 1 to 5% in different preparations. The specific extinction of the 'native' haemin *a* preparation was very low since the preparation contained much phospholipid and other lipid. These lipid impurities were much less readily removed from the haemin than from the porphyrin, largely because of the better chromatographic behaviour and solubility in HCl of the latter.

Porphyrin a from other tissues

Lemberg, Stewart & Bloomfield (1955) and Lemberg, Morell, Lockwood, Stewart & Bloomfield (1956) have measured the porphyrin derived from various rat and ox organs. Recent determinations on chicken heart and liver gave values of 20 and 21 mg./kg. wet wt. respectively, based on a specific extinction at 558 mμ of 27 for porphyrin *a*. The value for chicken heart is of the same order as that found for ox heart (19–22 mg./kg. wet wt.) or rat heart (24–27 mg./kg. wet wt.), but the value for chicken liver is unusually high compared with those for rat and ox livers, in which the concentration of porphyrin is less than 30% of that in their respective heart tissue.

The method of analysis on chicken tissues differed slightly from those done on other tissues by Lemberg, Stewart & Bloomfield (1955). Instead of minced tissues being used, the chicken tissues were homogenized in a high-speed blender with 0·1M-sodium acetate buffer, pH 5·6, and the residue containing the haematin *a* was recovered by centrifuging at 10 000 *g*. After resuspension in acetate buffer and recentrifuging the residue was extracted with cold acetone several times and then with acid–acetone. The method was therefore essentially the same as that used by the above authors. Porphyrin *a* of ratio band III/band IV of 2·4 was obtained from both chicken liver and heart.

Unlike animal tissues and bacteria, haematin *a* could not be extracted from yeast cells (*Saccharomyces cerevisiae*, *Torulopsis utilis*) by acetone–HCl even after the cells had been autolysed or lysed with toluene or $CHCl_3$. However, some success was achieved if the cells were lysed with pyridine and then extracted with acid–acetone. In this method pyridine was added to yeast paste to give a final concentration of 5% (v/v). After 5 hr. at 37° acid–acetone was added so that the acetone concentration was 80% (v/v), the water concentration 20% (v/v) and the pH 2·0. The haemin *a* and protohaemin were converted into the porphyrins, and the porphyrin *a* was purified by cellulose chromatography and by fractionation between HCl and ether. Porphyrin *a* with ratio band III/band IV of 2·24 was obtained, but because of gross contamination of the crude preparation with lipids the yields were not quantitative.

The concentrations of haemin *a* and protohaemin have been measured as their porphyrins in green peas (picked before the phase of starch accumulation), dried peas and wheat germ. The method involved extraction of the homogenized material with aq. acetone–water (4:1, v/v) to remove chlorophyll pigments, extraction of the haemins with acetone–HCl, conversion into the porphyrins, extraction of protoporphyrin with 4% HCl, and extraction of porphyrin *a* with 20% HCl from ethereal solution. Porphyrin *a* with ratio band III/band IV of 1·6–1·8 was obtained and contained traces of chlorophyll and carotenoid pigments. Green peas yielded about 0·35 mg. of porphyrin *a* and 2·0 mg. of protoporphyrin/kg. dry wt., or 0·022μg. of porphyrin *a* and 0·13μg. of protoporphyrin/seed. In dried peas 0·25μg. of protoporphyrin/seed was found but no porphyrin *a* could be detected. Wheat germ yielded 2·0 mg. of porphyrin *a* and 16 mg. of protoporphyrin/kg. wet wt. It would seem from these results that, on ripening and drying of peas, haemin *a* is lost. The very low concentration of haemin *a* in these tissues and the difficulty of achieving good spectroscopic purity of the porphyrin *a* makes the chemical determination of porphyrin *a* in plant tissues inaccurate and difficult.

DISCUSSION

The preparation of porphyrin *a* given in this paper is an improvement over earlier methods in two main respects: (1) Chromatography on cellulose-powder columns showed that earlier preparations produced variable amounts of a porphyrin which we have now shown to be derived from porphyrin *a* during the conversion into porphyrin by oxidation of the formyl group to carboxyl. Although this alteration product can be largely separated by thorough chromatography, the present method avoids this complication by destroying the oxidant in the acetic acid which causes the alteration. This finding is applicable to all formyl porphyrins or haemins. (2) Heat treatment of porphyrin *a* after the chromatography stage allows consistent improvement in specific extinction at 558 mμ from 8–10 to 20–24 when the porphyrin is extracted into 17% hydrochloric acid. It would seem that some of the lipid is very closely bound to the porphyrin (possibly by van der Waals forces) and that heat treatment alters this combination. The chromatography step precedes the heat treatment and hydrochloric acid-fractionation step because otherwise the porphyrin *a* becomes too strongly adsorbed on the cellulose for efficient purification.

Although the ratio of band III/band IV given for

the porphyrin *a* in the final step of the preparation described here was 2·36, a number of fractions in this and other preparations had ratios from 2·40 to 2·45. Throughout this study we have assumed that the higher this ratio the greater the spectroscopic purity of the porphyrin *a*. This assumption is based on the fact that any alteration to the formyl substituent, which is probably the most labile group in the molecule, lowers this ratio. Lemberg & Falk (1951) have pointed out that, where a second electrophilic group is introduced on a pyrrole ring vicinal to the other, the ratio of band III/band IV is lowered. Thus our assumption could be invalid if a third electrophilic group was present on a pyrrole ring between the two others in intact porphyrin *a* and was destroyed. The evidence that this is not so is given in the next paper.

Warburg & co-workers (Warburg & Gewitz, 1951; Warburg *et al.* 1955) have prepared crystalline haemin *a*. Their method required as starting material 64 horse hearts. We have not been able to work on this scale; smaller preparations in our hands were unsuccessful and did not give crystalline haemin *a*. This may have been due to the undesirability of scaling down this preparation. The scale of our preparation, which can conveniently be reduced if desired, is particularly suitable for study of the spectral properties of haemin *a* and derivatives which require relatively small quantities of haemin *a*. Conversion into the porphyrin at an early stage, as in our preparation, offers several advantages. In particular, the characteristic absorption spectra of the porphyrins, with their sharp and high absorption bands, provides a greater sensitivity for the detection of alterations to the prosthetic group or in searching for the possible presence of prosthetic groups whose spectra are closely related and which might account for differences in cytochromes *a* and a_3.

Our porphyrin *a* preparations show no evidence of containing two porphyrins with slightly different band positions. As pointed out by Morell, Barrett, Clezy & Lemberg (1959), the concentration of cryptohaemin *a* in ox heart is too low to represent the prosthetic group of either cytochrome *a* or a_3. The two spots found on chromatography of our porphyrin *a* esters on paper, the slower-running of which largely disappears on purification, could conceivably indicate a second form of porphyrin *a*. However, the effect of purification and the probable effect of lipids on the chromatographic behaviour of porphyrin *a* make interpretation of these chromatograms uncertain. That there is only one type of prosthetic group for cytochromes *a*, a_3 and a_1 is shown by the above studies on mammalian tissues and by our finding, for cytochrome a_1 (Morell *et al.* 1959), that porphyrin *a* with spectral characteristics identical with those from these tissues could be extracted from *Proteus vulgaris*.

Our purest porphyrin *a* of specific extinction 20–24 has been calculated in this paper to be 85% pure. The residual impurity accompanies the porphyrin into 15 or 20% hydrochloric acid or into aqueous borate buffers. The lipophilic nature of porphyrin *a* or haemin *a* derived from the substituent long alkyl group or groups (see the next paper) adds to the difficulty of removing lipid impurity. Attempts to crystallize our material either as the porphyrin or haemin, with various solvents, were unsuccessful.

SUMMARY

1. A method of purifying porphyrin *a* of high spectroscopic purity and approximately 85% absolute purity from ox heart is described and the method given for its conversion into haemin *a*.

2. During the conversion of haemin *a* or other formyl haemins into their respective porphyrins, special treatment of the acetic acid solvent is often necessary to prevent oxidation of the formyl group to carboxyl.

We wish to thank Dr R. Lemberg, F.R.S., for his interest in this work and his encouragement and Miss M. Stewart and Miss L. Clark for much able assistance. This work was done under grants from the National Health and Medical Research Council of Australia.

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The Prosthetic Group of Cytochrome Oxidase

2. CHEMISTRY OF PORPHYRIN *a*

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(*Received* 4 *July* 1960)

Early chemical studies relating to porphyrin *a*, the porphyrin derived from the prosthetic group of cytochrome oxidase, have been reviewed by Lemberg (1953), where the evidence is discussed in support of the presence in this molecule of a formyl group and an unsaturated side chain conjugated with the ring system. Analytical figures obtained from haemin *a* (Warburg & Gewitz, 1951) have suggested the presence of an alkyl side chain in haemin *a*, and this has been confirmed by the molecular-weight determinations by other workers (Lemberg, 1953; Granick, 1952). Warburg, Gewitz & Völker (1955) formulated this alkyl chain as an extended vinyl group.

Haemin *a* has been degraded by the resorcinol-melt treatment (Warburg & Gewitz, 1953; Warburg *et al.* 1955) to yield, after removal of iron, a deuteroporphyrin-like compound which was called cytodeuteroporphyrin. However, bromine substitution indicated that cytodeuteroporphyrin differed from a true deuteroporphyrin by containing three and not two unsubstituted β positions, and recently Marks, Dougall, Bullock & Macdonald (1959) have confirmed this and established the structure of cytodeuteroporphyrin as I, by a direct synthesis of the molecule.

It is well established that a formyl group and an unsaturated side chain conjugated with the tetrapyrrolic ring system would be removed from a haemin by resorcinol-melt treatment, but the nature of the third substituent removed from haemin *a* during the conversion into cytodeuterohaemin has not been determined.

Evidence has been presented (Barrett, 1959) for the presence of a hydroxyl group in porphyrin *a*. Since it has been established (Schumm, 1929) that α-hydroxyethyl groups are removed by the resorcinol-melt procedure, it was decided to investigate the possibility that this group was the third substituent removed during the formation of cytodeuterohaemin. The results of this work together with other aspects of the chemistry of porphyrin *a* are given in this paper. Preliminary reports covering part of this work have been published elsewhere (Clezy & Barrett, 1959; Barrett & Clezy, 1959).

METHODS AND MATERIALS

Absorption spectra. Measurements were made with a Hilger Uvispek spectrophotometer or with a Beck-Hartridge reversion spectroscope as indicated in the tables.

Solvents. Diethyl ether (not containing reducing agent) was used as supplied commercially. $CHCl_3$ was distilled to free it from trace metals. Acetone, used as a solvent for oxidation, was distilled over $KMnO_4$. Pyridine was dried over KOH and distilled.

Chromatographic alumina. 'Aluminium oxide for chromatographic adsorption analysis' (British Drug Houses Ltd.) was used. This was washed with water until the washings were neutral to litmus paper, dried overnight at 60° and sieved through muslin.

Cellulose-powder chromatography. The preparation of the columns is described by Morell, Barrett & Clezy (1961). For 2–5 mg. amounts of porphyrin, columns 1 cm. × 40 cm. were used.

Paper chromatography. The method of Chu, Green & Chu (1951) with slight modifications was used. The methyl esters of the porphyrins were run on separate sheets of Whatman no. 1 paper in kerosene (commercial grade, Shell Co. Ltd.)–chloroform (4·0:2·6) solvent 1, and kerosene–propanol (5·0:1·0) solvent 2.

Introduction and removal of iron. Unless otherwise stated iron was introduced into porphyrins and removed from haemins by methods described in the previous paper (Morell *et al.* 1961).

Oxime reaction. A small amount of porphyrin was dissolved in pyridine–water (4:1, v/v). The solution was heated with excess of hydroxylamine hydrochloride for 20 min. in a boiling-water bath.

Bisulphite reaction. A small amount of porphyrin was dissolved in a pyridine–water (4:1, v/v) and to this solution was added a few crystals of $NaHSO_3$. The mixture was shaken thoroughly and kept for $\frac{1}{2}$ hr. at room temperature (cf. Parker, 1959). The reaction is reversed by heat.

Acetate test for hydroxyl groups. This is described by Barrett (1959).

Chromium trioxide reagent for oxidation. The reagent (Jones reagent; Bowden, Heilbron, Jones & Weedon, 1946) was prepared as described by Djerassi, Engle & Bowers (1956).

Model porphyrins. Porphyrins, used as model compounds throughout this investigation, were prepared as follows: 2:4-diacetyldeuteroporphyrin dimethyl ester (Fischer & Zeile, 1929); monoacetyldeuteroporphyrin dimethyl ester (obtained as a by-product of the 2:4-diacetyldeuteroporphyrin preparation and separated from it by alumina chromatography); monoformyldeuteroporphyrin

dimethyl ester (Fischer & Wecker, 1942); chlorocruoroporphyrin (2-formyl-4-vinyldeuteroporphyrin) dimethyl ester (Lemberg & Parker, 1952); 2:4-diformyldeuteroporphyrin dimethyl ester (Lemberg & Parker, 1952). Monovinylmonoacetyldeuteroporphyrin was prepared by the partial reduction of diacetyldeuteroporphyrin with $NaBH_4$ and the subsequent dehydration of the monohydroxyethylmonoacetyldeuteroporphyrin formed at the reduction step. Monohydroxyethylmonovinyldeuteroporphyrin was prepared either by the reduction of monovinylmonoacetyldeuteroporphyrin or by partial hydration of protoporphyrin. Cryptoporphyrin *a* dimethyl ester was prepared by Miss J. Parker (Parker, 1959).

Porphyrin a. Porphyrin *a* used in this investigation was prepared by the methods described in the preceding paper (Morell *et al.* 1961).

Hydrochloric acid solutions. These are described in the preceding paper (Morell *et al.* 1961).

EXPERIMENTAL AND RESULTS

Acetal formation with carbonyl porphyrins

About 0·5 mg. of the porphyrin was dissolved in 5 ml. of methanol containing 3–4 drops of 10N-HCl, and the solution was boiled in a water bath for 5 min. After the solution was cooled, the porphyrin was returned to ether by using aqueous sodium acetate to neutralize the HCl. The ethereal solution was then washed free of acetic acid.

Porphyrins containing formyl groups conjugated with the ring system readily form dimethylacetals which show marked shifts of absorption maxima towards the blue end of the spectrum compared with the original formyl absorption bands (Table 1). Monoacetyl- and diacetyl-deuteroporphyrin exhibited little spectroscopic change when they were treated with methanolic HCl (see Table 1).

The acetal test allows distinction to be made between formyl and acetyl porphyrins, and since methylacetals can be hydrolysed by 5% HCl this group can be used to protect a formyl substituent in reactions carried out at neutrality.

Porphyrin a *acetate*

Porphyrin *aα* (8·5 mg., Lemberg & Stewart, 1955) was dissolved in 9 ml. of pyridine containing 1·5 ml. of acetic anhydride, and the mixture kept at room temperature for 3 days. The porphyrin was then returned to ether and the pyridine removed by washing with 1% HCl. Methylation of the porphyrin carboxyl groups with diazomethane followed, and, after freezing out water from the ethereal solution at −15°, the porphyrin mixture was applied to a cellulose column in a solvent mixture containing 2 ml. of $CHCl_3$ and 4 ml. of light petroleum (b.p. 60–80°). The porphyrin was eluted with the same solvent mixture, by which porphyrin *a* ester acetate (early fractions) was separated from porphyrin *a* ester and degradation products. Paper chromatography in solvent system 2 was used to determine which fractions contained the acetate. A yield of 3 mg. of porphyrin *a* ester acetate was obtained; compared with the parent compound the acetate showed an average shift to the blue of its absorption bands of 2 mμ. This spectral change was reversed on hydrolysis of the acetate.

From experiments in which the acetylation of porphyrin *aβ* and porphyrin *aα* were carried out in parallel it was concluded that porphyrin *aβ* acetylated more rapidly than did the α form. In general porphyrin *a* is not acetylated as readily as other formyl porphyrins containing α-hydroxyl groups which we have examined.

Oxidation of porphyrin a *and its derivatives*

The spectroscopic data relating to this series of experiments are shown in Table 2.

Chromium trioxide oxidation of porphyrin a *ester.* Porphyrin *a* dimethyl ester (1 mg.), dissolved in 5 ml. of acetone, was gassed with N_2 for 5 min., one drop of the CrO_3–H_2SO_4 reagent was added, and the oxidation allowed to proceed under N_2 for 1 min. The porphyrin was extracted into ether and the acetone washed out with water containing a little $FeSO_4$. Extraction of the ethereal solution with an aq. 1% Na_2CO_3 soln. separated a small amount of an acidic oxidation product from the major non-acidic oxidation product.

The non-acidic oxidation compound showed carbonyl properties, and from an examination of the shift in spectrum (greater than for one formyl group) on oxime formation and from the band positions of its dimethylacetal (to the red of the acetal of porphyrin *a*) it was judged this compound contained a ketonic group as well as a formyl group. The acidic oxidation product obtained from porphyrin *aα* also exhibited carbonyl properties (oxime reaction), and, moreover, it was demonstrated that this group was ketonic in character as negative bisulphite and acetal tests were obtained.

Formation of porphyrin a *acid from porphyrin* a *acetate.* Oxidation of porphyrin *a* acetate by the method given

Table 1. *Absorption spectra of carbonyl porphyrins before and after treatment with methanol–hydrochloric acid*

The solvent was ether. Spectra were determined with the spectrophotometer; Δ refers to the average shift in mμ of the four bands. Cryptoporphyrin *a* is a formyl porphyrin with one unsaturated side chain in which the double bond is conjugated to the ring (cf. Parker, 1959).

Porphyrin	Maxima (mμ) Before treatment				After treatment				Δ
Monoformyldeuteroporphyrin	640·5	578	550·5	510·5	623	568	526·5	497	16
2:4-Diformyldeuteroporphyrin	648	593	557·5	521	625	570	528	498·5	25
Chlorocruoroporphyrin (2-formyl-4-vinyldeuteroporphyrin)	642	583	555	514·5	628	573	532	500·5	15
Cryptoporphyrin *a*	642	584	555	515	628	574	532	501	15
Porphyrin *a*	646	582	558	517	632	571·5	538·5	502·5	15
Monoacetyldeuteroporphyrin	634·5	576	543	505·5	633	576	542·5	503	1
2:4-Diacetyldeuteroporphyrin	639	586	546·5	512·5	638·5	584	545·5	510·5	1

Table 2. *Absorption maxima of porphyrin* a, *porphyrin* a *alcohol, and the oxidation derivatives of porphyrin* a

The solvent was ether. All measurements were made with the spectrophotometer.

Porphyrin	Absorption maxima of porphyrin (mμ)					Absorption maxima of the dimethylacetal (mμ)					Absorption maxima of the acetate (mμ)					Oxime shift of original porphyrin-average of four bands
	I	II	III	IV	III/IV	I	II	III	IV	III/IV	I	II	III	IV	III/IV	
Porphyrin *a*	646	582	558	517	2·30	631	571	538	503	0·88	645	581	556	516·5	2·0	7·6
Porphyrin *a* alcohol	628	572	536	502	0·78	No acetal formed					626	570·5	534	500	0·74	0·0
Porphyrin *a* acid	642	579	552	511	1·79	No acetal formed					640	577	549	509	1·36	0·0
Non-acid oxidation product of porphyrin *a* ester	648	590	558	519	1·25	636	582	542	507	0·71	No acetate formed					9·7
Acidic oxidation product of porphyrin *a* ester	644	587	552	514	0·80	No acetal formed					No acetate formed					3·9
Oxidation product of porphyrin *a* acid	644	588	552	514	0·78	No acetal formed					No acetate formed					3·7

above yielded an acidic compound which showed no carbonyl properties. Hydrolysis of the acetate group, by keeping the material in 25% HCl overnight, gave a compound which can be formally derived from porphyrin *a* itself by the oxidation of the formyl group to a carboxylic acid group. This compound we propose to call porphyrin *a* acid.

Oxidation of porphyrin a *acid.* Oxidation of porphyrin *a* acid by the CrO_3 method gave a compound which was identical with the acidic oxidation product obtained from porphyrin *a* itself.

Oxidation of oximes of formyl porphyrins. Oxidation of the oximes of porphyrin *a*, hydrated porphyrin *a* (in which the double bond conjugated to the ring has been hydroxylated) and 2-formyl-4-hydroxyethyldeuteroporphyrin with CrO_3 gave compounds which showed a shift of their absorption bands towards the red. From a comparison of the relative shifts (Table 3), it is evident that two α-ketonyl groups are present in oxidized hydrated porphyrin *a* oxime.

Oxidation of porphyrin a *alcohol* (–CHO *reduced to* $-CH_2 \cdot OH$) *by potassium permanganate.* Porphyrin *a* alcohol (4·7 mg.) in 20 ml. of acetone, heated under reflux in a water bath, was oxidized with $KMnO_4$ (5 × molar proportion) in acetone. The $KMnO_4$ solution was added over 2–3 min. After cooling, the acetone solution was centrifuged to remove precipitated MnO_2, the porphyrin was extracted into ether, and the acetone removed by frequent washing with water. The porphyrin mixture was fractionated by chromatography on cellulose. Any re-formed porphyrin *a* or porphyrin *a* alcohol was eluted with ether or acetone; the new formyl porphyrin formed by the oxidation of the 'vinyl' group usually required the addition of methanol to the acetone to elute it and always travelled down the column behind porphyrin *a* and porphyrin *a* alcohol.

The porphyrin *a* was derived by oxidation of the hydroxymethyl group of porphyrin *a* alcohol, an oxidation possibly brought about by precipitated MnO_2, since we have shown (Barrett & Clezy, 1959) that hydroxymethyl groups are little affected by $KMnO_4$ in acetone. This was supported by the observation that the yield of porphyrin *a* was noticeably greater in preparations containing most lipid in which cases larger amounts of MnO_2 were generated. To avoid this type of oxidation porphyrin *a* alcohol can be acetylated.

We have shown elsewhere (Barrett & Clezy, 1959) that the presence of a formyl group in the porphyrin molecule inhibits the oxidation of a double bond conjugated to the ring, although R. Lemberg (unpublished work) has obtained from porphyrin *a*, in very small yield, a diformyl porphyrin by this method. However, prior reduction of the formyl group in porphyrin *a* is essential for a practical oxidation of the 'vinyl' group to a formyl group.

Reduction of the formyl group in porphyrin a

With sodium borohydride (*porphyrin* a *alcohol*). In a solution containing 1 mg. of porphyrin *a* in 20 ml. of ether was suspended 20 mg. of $NaBH_4$, and the mixture kept overnight. The excess of $NaBH_4$ was then decomposed with 0·5% HCl, leaving the reduction product, porphyrin *a* alcohol, in the ether. Unlike other carbonyl porphyrins which retain the four absorption bands of the neutral spectrum, porphyrin *a* on the addition of $NaBH_4$ to the ether shows a two-banded spectrum, reminiscent of that of a metal (eg. Na) complex. The dilute acid used to destroy the excess of reducing agent is sufficient to remove the

metal from the porphyrin *a* alcohol. The dimethylacetal of porphyrin *a* is not reduced by $NaBH_4$, porphyrin *a* itself being recovered after mild acid hydrolysis of the acetal group.

Wolff–Kishner reduction. Porphyrin *a* (20 mg.), moistened with 2–3 ml. of ethanol, was dissolved in 50 ml. of diethylene glycol. To this solution, 200 mg. of hydrazine sulphate and 400 mg. of KOH dissolved in 3 ml. of water were added, and the mixture was heated in an oil bath under a water condenser for 1 hr. The water was then run out of the condenser jacket, and the temperature in the reaction flask allowed to rise to 190–200° where it was maintained for a further hour. After cooling, the reaction mixture was poured into 400 ml. of water, acidified with HCl and the porphyrin extracted into ether. The methyl ester of this porphyrin was purified by alumina chromatography. The yield was 30%.

By this procedure the formyl group in porphyrin *a* was reduced to a methyl group giving a product (I) which was spectroscopically similar to monohydroxyethylmonovinyldeuteroporphyrin. This reduction product exhibited no carbonyl properties (no oxime reaction) but was shown by acetylation to contain a hydroxyl group; this hydroxyl group could be oxidized by CrO_3–H_2SO_4 to a porphyrin (III) spectroscopically similar to monoacetylmonovinyldeuteroporphyrin (Table 4).

Table 3. *Absorption spectra of porphyrin oximes and their oxidation products*

The solvent was ether. Absorption spectra were determined with the spectrophotometer. Δ refers to the average shift of the four bands after oxidation.

Compound	Absorption maxima of porphyrin (mμ) I	II	III	IV	Ratio band III/ band IV	Δ (mμ)
2-Formyl-4-hydroxyethyldeuteroporphyrin oxime	632	573	550	505	1·24	—
2-Formyl-4-hydroxyethyldeuteroporphyrin oxime oxidized	634	582	555	509	0·83	5
Porphyrin *a* oxime	638	578	547	509	1·46	—
Porphyrin *a* oxime oxidized	640	585	548	513	0·83	3
Hydrated porphyrin *a* oxime	632	576	543	518	—	—
Hydrated porphyrin *a* oxime oxidized	638	585	551	503	0·93	7

Table 4. *Absorption maxima of porphyrin* a *derivatives and model compounds*

The solvent was ether. All measurements were made with the spectrophotometer.

	Compound	Substituents at the 2, 4 and 8 positions of the porphyrin ring				Absorption maxima (mμ) I	II	III	IV	Ratio band III/ band IV
I	Wolff–Kishner reduction product of porphyrin *a*		CH(OH)·R′	CH:CHR″	CH_3	626	572	532	499	0·80
II	Monohydroxyethylmonovinyldeuteroporphyrin	or	CH(OH)·CH_3 CH:CH_2	CH:CH_2 CH(OH)·CH_3	CH_3	626	571	533	499	0·60
III	Oxidation compound of I		CO·R′	CH:CHR″	CH_3	633	579	546	507	0·85
IV	Monoacetylmonovinyldeuteroporphyrin	or	CO·CH_3 CH:CH_2	CH:CH_2 CO·CH_3	CH_3	637	580	547	508	0·90
V	Resorcinol-melt product of the non-acid product of porphyrin *a* ester		CO·R′	H	H	—	—	545	505	1·0
VI	Resorcinol-melt product of III		CO·R′	H	CH_3	633	575	544	504	0·95
VII	Monoacetyldeuteroporphyrin	or	CO·CH_3 H	H CO·CH_3	CH_3	633	576	543	505	1·10
VIII	Dehydration product of porphyrin *a*		CH:CHR′	CH:CHR″	CHO	648	584	562	520	2·0
IX	Dehydration product of porphyrin *a* acid		CH:CHR′	CH:CHR″	CO_2H	645	582	553·5	513	1·5
X	Dehydration product of porphyrin *a* alcohol		CH:CHR′	CH:CHR″	CH_2·OH	633	579	540·5	505	0·80
XI	Porphyrin *a* hydrated		CH(OH)·R′	CH(OH)·R″	CHO	643	579	554·5	512	1·5
XII	Porphyrin *a* alcohol hydrated		CH(OH)·R′	CH(OH)·R″	CH_2·OH	628	572	536	502	0·78
XIII	Monoformylmonohydroxyethyldeuteroporphyrin		CHO	CH(OH)·CH_3	CH_3	639	580	554	514	1·68
XIV	Cytodeuteroporphyrin*			—		619	566	530	496	0·53

* Derived from the haemin obtained from the resorcinol-melt treatment of haemin *a*. Solvent, chloroform.

Dehydration of porphyrin a and its derivatives

The classical method of dehydration by heating *in vacuo* was not successful with porphyrin *a* or its derivatives as much polymerization occurred. Hence other methods of dehydration were examined and the following procedures were found to dehydrate satisfactorily porphyrin *a* acid and porphyrin *a* alcohol. Porphyrin *a* itself appeared to be more resistant to dehydration than these two derivatives, but the second procedure yielded a dehydration product from porphyrin *a*. Spectroscopic data are given in Table 4.

Dehydration by heating in pyridine with toluene-p-sulphonyl chloride. Porphyrin (1 mg.) was heated in 4 ml. of pyridine containing 20 mg. of toluene-*p*-sulphonyl chloride on a boiling-water bath for 7 hr. The porphyrin was then extracted into ether and the pyridine removed by repeated extraction with 1% HCl.

Dehydration by heating with toluene-p-sulphonic acid in benzene. Porphyrin ester (1 mg.) was heated in 5 ml. of benzene containing 2 mg. of toluene-*p*-sulphonic acid for 6 hr. For porphyrins such as haematoporphyrin, which contained no electronegative side chains, dehydration was complete in this time. With porphyrin *a* and its derivatives the benzene solution after cooling was poured on to a cellulose column, the column was washed with light petroleum and then ether, the latter eluting porphyrin *a* from which the hydroxyl group has been removed.

By these methods porphyrin *a* acid and porphyrin *a* have been dehydrated to give compounds which no longer contain hydroxyl groups and which show a shift in absorption maxima of 2–3 mμ towards the red end of the spectrum. Porphyrin *a* alcohol can also be dehydrated to a compound which shows a similar spectroscopic shift but this compound still contains a hydroxyl group, the hydroxymethyl group formed by the reduction of the formyl group in porphyrin *a*.

Although it seems that α-hydroxyethyl porphyrins are dehydrated more readily under acidic than basic conditions, the ease of this reaction is governed primarily by the nature of the other substituent groups. In a series of porphyrins, (I) monohydroxymethyl-, (II) monovinyl-, (III) monocarboxy-, (IV) monoacetyl-, and (V) monoformyl-monohydroxyethyldeuteroporphyrin, the relative rate of dehydration can be indicated as $I = II \gg III = IV > V$. Thus, in accord with expectations, the ease of dehydration decreases as the electronegative nature of the substituent increases.

Hydration of vinyl-substituted porphyrins

The porphyrin (2 mg.) was treated with 1 ml. of HBr in acetic acid [50% (w/v) HBr, British Drug Houses Ltd.] in a stoppered tube overnight. To this was then added 10 ml. of 5% HCl and the solution, after gassing with N_2, was left at room temperature for 24 hr. The hydrated porphyrin was then extracted into ether over an aq. 10% sodium acetate soln.

As would be expected both chlorocruoroporphyrin and porphyrin *a* were hydrated more slowly than protoporphyrin.

Resorcinol-melt treatment of haemin a derivatives at 200°

In an unpublished investigation carried out in this Laboratory we have observed that α-ketonyl and carboxyl groups in conjugation with porphyrin ring are not removed in the resorcinol melt if this is carried out below 200°, whereas hydroxyethyl, hydroxymethyl, formyl and vinyl groups are thereby removed from the porphyrin. This greater stability of the acetyl groups has been utilized in the following experiment.

A mixture of haemin and five times its weight of resorcinol was heated at 190–195° for 15 min. After cooling, the reaction mixture was dissolved in ether, and this solution was washed well with 1% HCl which removed yellow by-products. After the ethereal solution had been dried (– 15° overnight), the solvent was removed *in vacuo* to leave the crude haemin which was converted into the corresponding porphyrin by using $FeSO_4$ in acetic acid (100 moles of $FeSO_4$/mole of porphyrin). The crude porphyrin was purified by acid fractionation and by alumina chromatography of the dimethyl esters.

The non-acidic compound obtained by the oxidation of porphyrin *a* dimethyl ester, and that (III, Table 4) obtained by the oxidation of the Wolff–Kishner reduction product of porphyrin *a* were converted into their respective haemins and subjected to the resorcinol-melt treatment. Both (V and VI, Table 4) yielded porphyrins spectroscopically similar to monoacetyldeuteroporphyrin (VII, Table 4) but differed significantly from this model porphyrin in R_F values (solvent 1; compounds V and VI, 0·52; compound VII, 0·49: solvent 2; compounds V and VI, 0·68; compound VII, 0·55) and in HCl number (compounds V and VI, approx. 12; compound VII, 4).

Comparison of the relative rate of formation of porphyrin aβ from porphyrin aα and the hydroxylation of protoporphyrin in hydrochloric acid

It has been shown by Lemberg & Stewart (1955) that porphyrin *a* is transformed into the β form spectrally identical with porphyrin *aα* by standing in dil. HCl. Since the vinyl groups of protoporphyrin are hydrated under these conditions, we considered the possibility that the $\alpha \rightarrow \beta$ transformation was due to the hydroxylation of a double bond, in one of the alkyl side chains, not conjugated to the porphyrin ring. The respective porphyrins (2 mg.) were kept in 20 ml. of 2, 5 and 20% HCl at room temperature for 1–4 days. The porphyrins were extracted into ether, and the ether solution was analysed for the content of porphyrin *aβ* in the one series and for monovinylmonohydroxyethyldeuteroporphyrin and haematoporphyrin in the other. Porphyrin *aβ* was readily formed from porphyrin *aα* in 2 and 5% HCl, more slowly in higher concentrations. The hydroxylation of the vinyl group was similarly more efficient at the lower concentrations of HCl. However, there was a very marked difference in the rate of formation of porphyrin *aβ* and the hydroxylation of the vinyl groups. After 24 hr. in 5% HCl there was a 50% conversion into porphyrin *aβ* and by 3 days this was complete, whereas after 14 days in 5% HCl only 10% of the protoporphyrin had been hydrated. Though protoporphyrin is not a strict model the large difference in rates suggest strongly that the addition of water to a double bond in an alkyl side chain is not the explanation of the $\alpha \rightarrow \beta$ transformation.

DISCUSSION

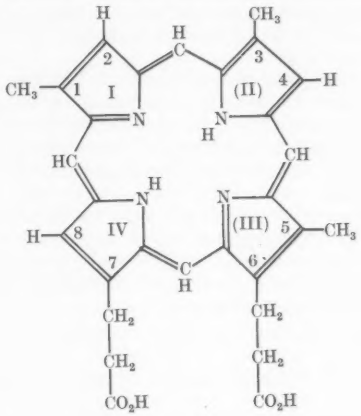
Analytical determinations on haemin *a* (Warburg & Gewitz, 1951) indicated the presence of at least six oxygen atoms in this molecule. Five of

these atoms are accounted for by the two propionic acid side chains and by the formyl group, and it seemed likely that the sixth oxygen atom provided an activating influence which resulted in the loss of the third and unknown substituent during the conversion of porphyrin *a* into cytodeuteroporphyrin. We have therefore attempted to characterize the group containing this sixth oxygen atom.

The magnitude of shift of absorption bands to the blue upon oxime formation can be taken as a measure of the number of carbonyl groups in the porphyrin molecule. From Table 5 it is apparent that the oxime shift observed with porphyrin *a* is of the same order as those found for other monoformyl porphyrins and is smaller than for diformyldeuteroporphyrin and diacetyldeuteroporphyrin. This indicates that neither a second formyl group nor an acetyl group conjugated with the ring system is present in porphyrin *a*. Moreover, there is additional evidence for excluding from porphyrin *a* an acetyl group conjugated with the ring system. Porphyrin *a* is readily reduced by sodium borohydride but porphyrin *a* dimethylacetal is spectroscopically unaltered by this reagent. It has been shown above that acetyl porphyrins do not form acetals under the conditions quoted in this paper and yet are readily reduced by sodium borohydride; that no spectroscopic change occurs when porphyrin *a* dimethylacetal is treated with borohydride argues against the presence in the porphyrin *a* molecule of an acetyl group conjugated with the ring system. In addition, we have shown in experiments with model compounds that acetyl groups are substantially unaffected by the resorcinol-melt procedure when it is carried out below 200°. Thus it can be concluded that the formyl group is the only carbonyl substituent, conjugated with the tetrapyrrolic ring system in porphyrin *a*.

Experiments reported in this paper show, however, that a ketonyl group in conjugation with the porphyrin ring system can be introduced into porphyrin *a* and porphyrin *a* acid by oxidation with chromium trioxide. Since these ketonyl compounds no longer contained hydroxyl groups (acetylation test) we assume that the new carbonyl group was formed by the oxidation of the hydroxyl group shown to be present in porphyrin *a* (Barrett, 1959). In addition it has been demonstrated in this paper that this hydroxyl group can be removed from porphyrin *a* and its derivatives by dehydration procedures, the resulting compound showing shifts in absorption maxima to the red end of the spectrum indicating the formation of a carbon–carbon double bond conjugated with the porphyrin ring system. An examination of the spectrum of porphyrin *a* shows that it lies 4 mμ (average of the four bands) towards the red compared with the spectrum of monoformylmonohydroxyethyldeuteroporphyrin. This difference of 4 mμ can be accounted for entirely by the presence, in addition to the hydroxyalkyl and formyl groups, of the group —CH:CHR known to be present in porphyrin *a*.

The synthetic work of Marks *et al.* (1959) has established the structure of cytodeuteroporphyrin as (I) and shown that the unsubstituted β-pyrrolic carbon atoms present in this molecule occur at positions 2, 4 and 8. Earlier Lemberg (1953) from the spectroscopic properties of porphyrin *a* deduced that the formyl group and the unsaturated side chain must occupy opposite pyrrolic rings. This



(I)

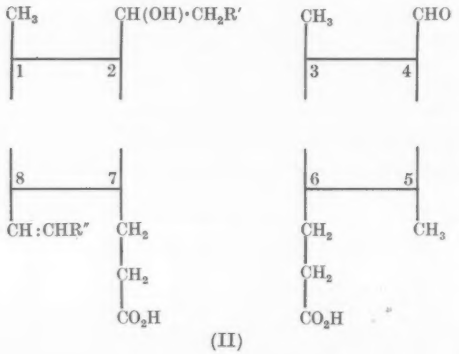
Table 5. *Absorption maxima of carbonyl porphyrins and their oximes in pyridine*

Determinations were made with a Beck–Hartridge reversion spectroscope. Δ refers to the average shift of the four bands.

Porphyrin	Porphyrin bands (mμ)				Oxime bands (mμ)				Δ
Monoformyldeuteroporphyrin	638·4	583·4	551·5	513·0	630·0	578·2	540·9	504·8	8·5
2:4-Diformyldeuteroporphyrin	648·3	594·6	560·5	525·4	638·9	586·7	548·3	512·5	10·7
Chlorocruoroporphyrin	642·7	587·6	556·3	517·2	635·8	583·1	544·9	509·6	7·6
Monoacetyldeuteroporphyrin	634·8	580·0	545·7	506·9	623·4	574·3	534·3	500·6	9·6
2:4-Diacetyldeuteroporphyrin	638·9	587·8	552·7	515·5	625·5	576·6	537·1	503·5	13·0
Porphyrin *a*	645·0	588·0	560·5	519·0	637·2	580·4	551·6	513·0	7·6

deduction is substantiated by the lowering of the ratio of band III/band IV of porphyrin *a* on hydration of the double bond (see Table 4). The formyl group and unsaturated side chain must then occupy positions 4 and 8. The hydroxyl group in porphyrin *a* must be so placed that on oxidation a keto group is produced which is conjugated with the porphyrin ring system. This limits its placement to either the γ-carbon atom of the extended vinyl chain or the α-carbon atom of the grouping at position 2. A decision between these two alternatives can be made on the available spectroscopic evidence. If the hydroxyl group were placed on the γ-carbon atom of the unsaturated chain, oxidation to a keto group or dehydration to produce a double bond would increase the electronegativity of this group. This would tend to raise the ratio of band III/band IV due to the presence of the formyl group in the opposite pyrrolic ring. However, oxidation of the hydroxyl group or its removal by dehydration lowers the ratio of band III/band IV in porphyrin *a* or porphyrin *a* acid (see Tables 2 and 4) indicating that the new electrophilic group has been introduced into a ring adjacent to the pyrrole ring carrying the formyl or carboxyl acid groups. Thus the hydroxyl group in porphyrin *a* is placed on the α-carbon atom of the group situated at position 2. On the basis of this evidence porphyrin *a* can be formulated as (II) or (III).

Piatelli (1960) has distinguished between these possibilities by identifying the pyrrolic acid (IV) as one of the oxidation products of porphyrin *a* nitrile, obtained by the dehydration of porphyrin *a* oxime. This indicates that the formyl group present in porphyrin *a* occurs on one of the pyrrolic rings carrying a propionic acid side chain and this, coupled with the structure given by Marks *et al.* (1959) for cytodeuteroporphyrin, has led us to propose (see Lemberg, Clezy & Barrett, 1959) formula (III) as the structure for porphyrin *a*.

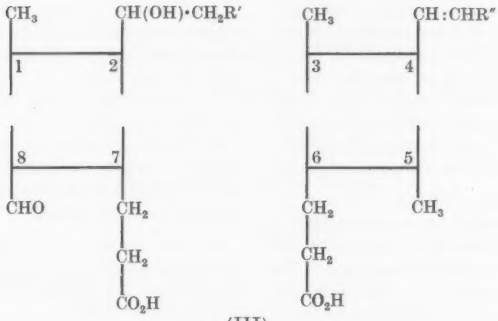


(II)

The Wolff–Kishner reduction of porphyrin *a* gave a compound spectroscopically similar to monohydroxyethylmonovinyldeuteroporphyrin. Oxidation of this compound by the Jones reagent gave a ketonyl porphyrin spectroscopically similar to monovinylmonoacetyldeuteroporphyrin. The production of both these compounds is in accord with structure (III) for porphyrin *a*. When this carbonyl porphyrin was subjected to the resorcinol-melt procedure another carbonyl porphyrin was obtained, which, although resembling monoacetyldeuteroporphyrin spectroscopically, differed from it in R_f and had a significantly higher HCl number than the model porphyrin. A ketonyl porphyrin of similar type was obtained by resorcinol melt of the non-acidic oxidation product of porphyrin *a* ester. Hence it must be assumed that the ketonyl porphyrins obtained from porphyrin *a* by the above procedures are not simple acetyl porphyrins, and it follows that the chain carrying the other group in porphyrin *a* itself is not simply an α-hydroxyethyl group. This would indicate that at least some of the 12–16 carbon atoms, which form the long alkyl side chain postulated by Warburg to account for his analytical figures from haemin *a*, are present in position 2. Warburg *et al.* (1955) had previously concluded that the unsaturated side chain was extended to include these extra carbon atoms since, after hydrogenation, haemin *a* no longer yielded crystalline material when subjected to the Schumm melt. Piatelli (1960) also has found evidence that points to the unsaturated side group in porphyrin *a* not being a simple vinyl group since the pyrrolic acid (V) was not amongst the oxidation products of hydrogenated porphyrin *a*. It would then seem that porphyrin *a* has two extended carbon chains, and this molecule can be represented by (III), where R′ and R″ are the two alkyl side chains the constituents of which have yet to be determined.

The formulation of the carbon–carbon double bond as —CH:CHR″ needs further confirmation. The appearance of an α-ketonyl group on this side chain after oxidation of hydrated porphyrin *a* oxime and the production of a formyl porphyrin by oxidative cleavage of this double bond both require the presence of an α-CH group at position 4, but there is no such evidence to confirm the presence of a hydrogen on the β-carbon atom. Also the carbon β to the porphyrin ring at position 2 may not simply be a methylene group, although at least one hydrogen must be so placed to account for the dehydration experiments.

In other reports from this Laboratory (Lemberg, 1955; Lemberg & Stewart, 1955) mention has been made of the $\alpha \rightarrow \beta$ conversion whereby the naturally occurring porphyrin $a\alpha$ of high HCl number is transformed by the action of dilute hydrochloric acid into porphyrin $a\beta$ of low HCl number. The



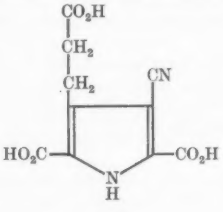
(III)

reason for this transformation is not yet understood but some progress has been made in determining the position of the groups involved in this conversion. The $\alpha \rightarrow \beta$ transformation does not occur in simple porphyrins so that it must be associated with one of the substituents at positions 2, 4 or 8, on the tetrapyrrolic ring. The formyl group is excluded, as porphyrin *a* alcohol can exist in either the α or β form. Since the formyl porphyrin obtained by oxidative scission of the unsaturated side chain in position 4 of porphyrin *a* alcohol can undergo the $\alpha \rightarrow \beta$ conversion, the locus of this transformation must lie in the hydroxyalkyl side chain at the 2 position of the ring system. Since α-hydroxyethyl porphyrins are known not to give this type of transformation, this is further evidence that this chain is extended beyond two carbons.

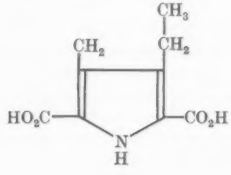
Both in partition studies and on chromatography porphyrin $a\beta$ behaves as a more polar compound than porphyrin $a\alpha$. That these properties are due to hydration of a double bond, isolated from the porphyrin ring, is unlikely, since the $\alpha \rightarrow \beta$ transformation persists after potassium permanganate treatment of porphyrin *a*. The comparative rates of formation of porphyrin $a\beta$ and the hydration of the vinyl groups of protoporphyrin in hydrochloric acid, although the latter compound is not a strict model because of conjugation of the double bonds to the ring system, support this conclusion.

The high HCl number of porphyrin *a* has been assumed to be due to the alkyl side chains present in this molecule. However, this cannot be the full explanation of this property as cryptoporphyrin *a*, a formyl porphyrin containing a large alkyl side chain (Parker, 1959), has an HCl number comparable with those of other monoformyl porphyrins. Further, the β form of porphyrin *a* has an HCl number of 5, which is very similar to that of monoformylmonohydroxyethyldeuteroporphyrin. Thus the high HCl number of naturally occurring porphyrin *a* must be a characteristic of the *a* form of this compound and not directly a result of the carbon chains in the molecule.

Morrison & Stotz (1959) and Morrison, Connelly, Petix & Stotz (1960), from a spectroscopic study of the pyridine haemochromes or haemin *a* and its derivatives, have concluded that a formyl group and a double bond conjugated with the porphyrin ring system are not sufficient by way of electronegative substituents to account for the spectroscopic properties of this molecule (cf., however, Lemberg *et al.* 1959). In particular they draw attention to the difference of 8·6 mμ (Lemberg & Falk, 1951) between the pyridine haemochrome of haemin *a* oxime and chlorocruorohaemin oxime, and suggest this is due to a keto group in the haemin *a* molecule, since Lemberg & Falk have shown that acetyl haemins do not react with hydroxylamine. Morrison *et al.* (1960) find support for the presence of a keto group in haemin *a* from the fact that dimedone, which reacts specifically with alde-



(IV)



(V)

hydes, yields a derivative with haemin *a* having a pyridine haemochrome at 558 mμ, whereas reduction of haemin *a* with sodium borohydride, which would reduce all carbonyl groups present, gives a haemin with a haemochrome at 552 mμ. These authors claim support for a second carbonyl grouping in haemin *a* from infrared spectroscopy. To explain these findings they propose that haemin *a* contains at position 2 the grouping $-\overset{\alpha}{C}H(OH)\cdot\overset{\beta}{C}O$.

It does not appear possible to reconcile such a formulation with the evidence put forward in this paper. In particular, it is difficult to explain the dehydration of porphyrin *a* and its derivatives in terms of such a structure. The absorption maxima of our dimethylacetal of porphyrin *a* lie about 2 mμ to the red of the corresponding bands of porphyrin *a* alcohol. This is an analogous situation to the dimedone-reduced haemin *a* comparison of Morrison *et al.* (1960). However, as mentioned earlier in this paper, porphyrin *a* dimethylacetal is quite stable to sodium borohydride, which indicates that any spectroscopic difference between it and the alcohol is not due to the presence of a carbonyl group in the molecule. Further, porphyrin *a* and chlorocruoroporphyrin have a very different orientation of substituents, and this may be the explanation of some of the spectroscopic differences between the two haemins reported by Morrison *et al* (1960).

As is shown in Table 2, the acetylation of porphyrin *a* and its derivatives result in a shift of absorption maxima towards the blue. This change is reversed upon hydrolysis of the acetate. Morrison *et al.* (1960) have also observed this type of spectroscopic shift when studying the pyridine haemochrome of haemin *a* and its acetate. This change must involve more than a simple acetylation of a hydroxyl group since model porphyrins hydroxylated on a carbon *a* to the ring system, e.g. haematoporphyrin or 2-formyl-4-hydroxyethyldeuteroporphyrin, do not exhibit this spectroscopic shift on acetylation. This shift on acetylation also occurs in the β form of porphyrin *a* and is therefore not contingent on this transformation. An explanation of this alteration and of the $\alpha \rightarrow \beta$ conversion must await the elucidation of the full structure of the substituent at the 2 position.

SUMMARY

1. Porphyrin *a* can be oxidized to a carbonyl derivative and dehydrated to a compound which contains a second carbon–carbon double bond. Since both these new functional groups are in conjugation with the pyrrolic ring system it is concluded that porphyrin *a* has a hydroxyl group on a carbon α to the porphyrin ring.

2. Evidence is presented which indicates that the α-hydroxyalkyl side chain is situated at the 2 position of the ring system and is of greater length than two carbon atoms. It has been shown that this side chain contains the locus of the $\alpha \rightarrow \beta$ transformation of porphyrin *a*.

3. From evidence presented in this paper porphyrin *a* can be described as 1:3:5-trimethyl-2-(α-hydroxyalkyl)-4-(β-alkylvinyl)-6:7-di-(β-carboxyethyl)-8-formylporphin.

We wish to thank Dr R. Lemberg, F.R.S., for his interest and encouragement throughout this work. We acknowledge support from the National Health and Medical Research Council of Australia.

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Studies in the Biosynthesis of Fungal Metabolites

3. THE BIOSYNTHESIS OF FUNGAL PERINAPHTHENONES*

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(*Received* 4 *August* 1960)

The existence of perinaphthenone derivatives as products of the fungal metabolism of sugars has recently been demonstrated for herqueinone ($C_{20}H_{20}O_7$), norherqueinone ($C_{19}H_{18}O_7$) and atrovenetin ($C_{19}H_{18}O_6$). Independent investigations of the colouring matters of *Penicillium herquei* led to the isolation of herqueinone by Stodola, Raper & Fennell (1951) and of herqueinone and norherqueinone by Galarraga, Neill & Raistrick (1955). A subsequent report of a study of the morphologically related species *P. atrovenetum* (Neill & Raistrick, 1956) described the isolation of the pigment atrovenetin.

In the course of these investigations, together with that of Barton, de Mayo, Morrison, Schaeppi & Raistrick (1956), the nature of the interrelationship of these pigments was recognized. Thus herqueinone is a monomethyl ether of norherqueinone, and this latter compound on treatment with zinc dust in acetic acid at room temperature yields deoxynorherqueinone, which is identical with atrovenetin. All three compounds were considered to be derivatives of 9-hydroxyperinaphthenone, but the first complete structure was not proposed until Barton, de Mayo, Morrison & Raistrick (1959) described chemical degradations which permitted the formulation of atrovenetin as (I), although the minor modification (II) remains a possible alternative (D. H. R. Barton, personal communication).

Although the structures of herqueinone and norherqueinone have not yet been fully determined, an essential step in arriving at a structure for atrovenetin involved its established identity with deoxynorherqueinone. Consequently, it is apparent that these three compounds possess a common carbon skeleton and it is therefore reasonable to assume that in each case this skeleton arises via the same biosynthetic mechanism.

Barton *et al.* (1959) suggested that the origin of the perinaphthenone nucleus (III) involves a poly-β-diketone intermediate (IV) (Fig. 1), as previously postulated for the genesis of certain other types of phenolic substances (Birch & Donovan, 1953). The ethereal side chain was assumed to be derived from a mevalonic acid (V) precursor.

Many instances of this mode of synthesis of fungal phenols have been demonstrated in recent years (Birch, 1957; Gatenbeck, 1958; Thomas, 1959), involving a head-to-tail linkage of acetate units, although the nature of the intermediates has not yet been elucidated. An alternative scheme of aromatic synthesis, leading, for example, to the amino acids phenylalanine and tyrosine, has been studied in considerable detail in mutant strains of *Neurospora crassa* and *Escherichia coli* (cf. Srinivasan, Shigeura, Sprecher, Sprinson & Davis, 1956). Acetate is not directly involved in this latter pathway, which utilizes a complex series of intermediates, including shikimic acid and prephenic acid, which arise from glucose via the hexose monophosphate shunt.

The simultaneous operation of both mechanisms of aromatic synthesis has recently been shown to take place in the biosynthesis of the flavanoid and anthocyanin pigments, quercetin (Watkin, Underhill & Neish, 1957; Geissman & Swain, 1957). cyanidin (Grisebach, 1957) and rutin (Shibata & Yamakazi, 1958). In each case, the experimental evidence supported the view that the two constituent benzene rings arose, one through the acetate and the other via the shikimic acid pathway. Thus despite the attractive nature of the acetate hypothesis as an explanation of the origin of the perinaphthenone nucleus, it was of interest to consider alternative possibilities in which one or more of the three rings arose from shikimic acid.

One such scheme involving the C_6–C_3–C_6 precursor (VI), shown in Fig. 2, could, by alternative cyclizations of the three acetate units, give rise either to the flavonol morin (VII) or the stilbene oxyresveratrol (VIII), which occur together in the wood of the osage orange, *Toxylon pomiferum* (Barnes & Gerber, 1955), or alternatively atrovenetin via the perinaphthenone (IX).

Rings B and C of the perinaphthenone would thus be required to arise from the precursor (VI), 2-(2:4-dihydroxyphenylpyruvoyl)-3:5-dioxohexanoic acid, by condensation of the carbon atoms 2 and 5

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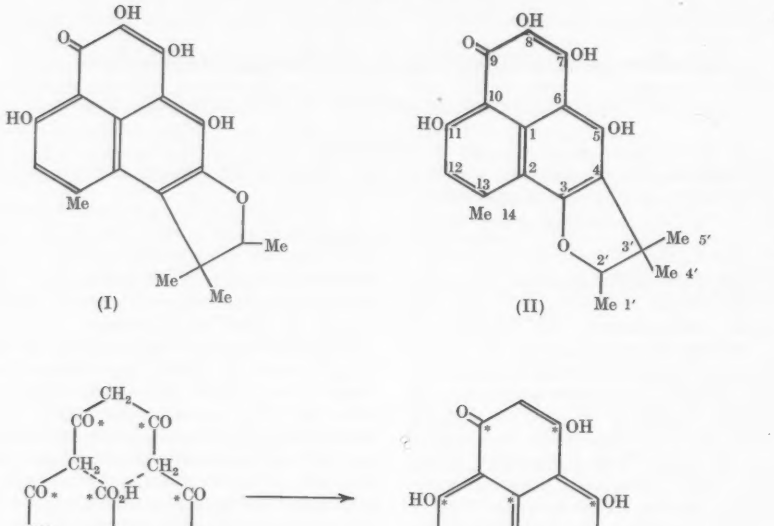

Fig. 1. Structure of atrovenetin and postulated biosynthesis. * Denotes acetate-carboxyl-derived carbon atoms.

of the hexanoic acid moiety with the phenyl group. However, the former condensation, giving rise to ring B, would be unlikely to proceed by direct oxidative coupling, owing to the unfavourable orientation of the two phenolic hydroxyl groups (Barton & Cohen, 1957). A more plausible coupling mechanism would involve oxidation of the resorcinol ring to a substituted phoroglucinol, which could then condense as its keto tautomer with the appropriate active methylene group. A subsequent decarboxylation step would leave only two acetate-carboxyl-derived carbon atoms in the perinaphthenone nucleus as shown, compared with seven if the pathway involved acetate exclusively (Fig. 1).

This difference provided a simple means of distinguishing between the alternative schemes. Thus if only acetate is involved, then a Kuhn–Roth oxidation of norxanthoherquein (X) would yield 1 mole equivalent of acetic acid containing one-seventh of its total activity; however, the alternative C_6–C_3–C_6 pathway would yield acetic acid containing approximately half the activity of the parent norxanthoherquein, assuming negligible incorporation of acetate into the phenylpyruvoyl moiety, as was shown to be the case for quercetin (Watkin *et al.* 1957).

The experimental approach was the same as that described in previous papers (Chaplen & Thomas, 1960; Thomas, 1961). Norherqueinone was selected in preference to herqueinone because of the relative ease of its isolation. Atrovenetin was considered to be less suitable, since its C_5 side chain is not readily cleaved on acid hydrolysis and the presence of two additional carbon-methyl groups would unnecessarily complicate the interpretation of the Kuhn–Roth oxidation data.

Accordingly, *P. herquei* was grown on a Raulin–Thom medium in surface culture as described by Galarraga *et al.* (1955) and sodium [1-^{14}C]acetate was added after incubation for 13 days at 24°. After a further 7 days' growth, the mycelium was harvested, washed and then dried. In a parallel experiment DL-[2-^{14}C]mevalonic acid lactone was fed to the mould. Both precursors gave rise to radioactive mycelia, which were continuously extracted with ether in a Soxhlet apparatus, yielding deep-red crystalline deposits in the extraction flasks, consisting of almost pure norherqueinone.

No attempt was made to recover norherqueinone from either the ether-soluble mycelial extracts or the *P. herquei* culture filtrates, so that calculations of the percentage incorporation of precursors yielded only minimum values: acetate, 1·8%; mevalonate, 0·75%.

In both cases, the active products were diluted to suitable working levels with inactive norherqueinone and purified by crystallization from acetic acid. Chemical degradations were then carried out according to the scheme illustrated in Fig. 3.

Thus the C_5 side chain was isolated as methyl *iso*propyl ketone (XI) by prolonged acid hydrolysis, leaving norxanthoherquein (X). The volatile ketone was absorbed in Brady's solution and the resulting precipitate of the 2:4-dinitrophenylhydrazone was purified by repeated crystallization from ethanol, before radioassay. Treatment of a further preparation of the ketone with alkaline iodine solution converted it into iodoform, which was also purified by crystallization from ethanol. This iodoform (presumed to arise from the non-*gem*-methyl group of the side chain) was found to be radioactive. An attempt to isolate a significant amount of the other product of iodine oxidation, probably *iso*butyric acid, as its *p*-bromophenacyl ester, was unsuccessful.

The norxanthoherquein resulting from acid hydrolysis, was separated by filtration and purified by repeated sublimation in a high vacuum. It was assayed for activity and then oxidized with chromic acid under Kuhn–Roth conditions, thereby producing an almost quantitative yield of acetic acid derived from the carbon-methyl group and a corresponding yield of carbon dioxide from the remaining 12 carbon atoms. The carbon dioxide was converted into barium carbonate for assay and the activity of the acetic acid was determined as its *p*-bromophenacyl ester. A further quantity of acetic acid was pyrolysed as its lithium salt to give acetone, which was subsequently caused to react with alkaline iodine solution and the resulting iodoform was purified as described above, before oxidation and assay as barium carbonate.

The observed activities of these degradation products (Table 1) were in agreement with the complete derivation of norherqueinone from acetate as postulated by Barton *et al.* (1959).

Thus sodium [1-^{14}C]acetate-derived norherqueinone contained approximately two-ninths of the total activity in the C_5 side chain and one-ninth in the methyl-substituted carbon atom of the norxanthoherquein nucleus, the methyl group itself being inactive. The activity of the carbon dioxide evolved during the Kuhn–Roth oxidation confirmed that the remaining 12 nuclear carbon atoms contained approximately two-thirds of the total activity as expected.

With regard to the origin of the C_5 side chain, alkaline-iodine oxidation of the methyl *iso*propyl ketone, obtained on acid hydrolysis, demonstrated that one-half of the total activity of this residue was present in the non-*gem*-methyl group, as required by hypothesis. Furthermore, the corresponding methyl *iso*propyl ketone moiety obtained from the DL-[2-^{14}C]mevalonic acid lactone-derived

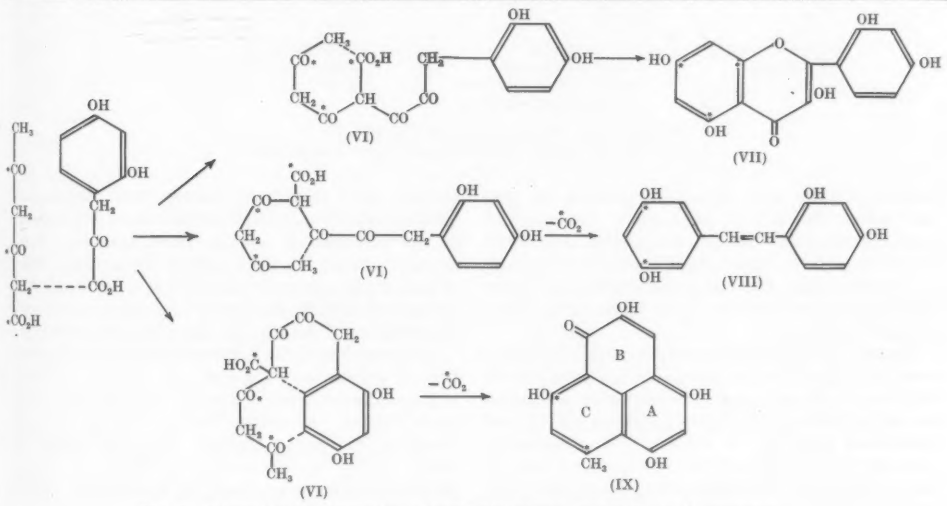

Fig. 2. Scheme for the formation of perinaphthenones from a C_6–C_3–C_6 precursor.
*, Denotes acetate-carboxyl-derived carbon atoms.

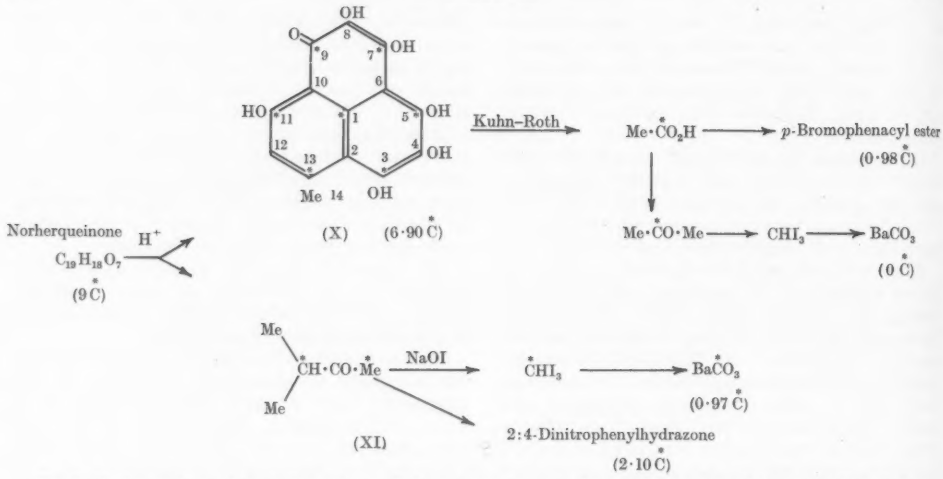

Fig. 3. Norherqueinone degradation scheme. Values shown in parentheses indicate the relative activities of the degradation products, calculated on the basis of a theoretical incorporation of nine acetate units into norherqueinone.

Table 1. *Activities of norherqueinone and its degradation products*

Precursor	Compound assayed	Norherqueinone-derived carbon atoms*	Activity (μc/mole) Found	Theory
$CH_3 \cdot {}^{14}CO_2H$	Norherqueinone	1–14, 1′–5′	1186	9 × 132†
	Norxanthoherquein	1–14	908	922
	Methyl *iso*propyl ketone dinitrophenylhydrazone	1′–5′	276	264
	p-Bromophenacyl acetate	13, 14	129	132
	$BaCO_3$	14	0	0
	$BaCO_3$	1–12	64	66
	$BaCO_3$	1′	128	132
[2-^{14}C]Mevalonic acid lactone	Norherqueinone	1–14, 1′–5′	116	—
	Norxanthoherquein	1–14	0	0
	Methyl *iso*propyl ketone dinitrophenylhydrazone	1′–5′	114	116

* Arbitrary numbering system as in (II) (Fig. 1).
† Based on the uniform incorporation of nine acetate units.

norherqueinone, was found to contain all the activity of the parent compound, the residual norxanthoherquein being completely unlabelled (Table 1). This confirmed the true isoprenoid nature of the side chain, the mevalonate precursor probably arising from acetate via β-hydroxy-β-methylglutaryl-coenzyme A.

Despite the fact that the acetate origin of the norxanthoherquein nucleus is based only on the results of Kuhn–Roth oxidation, it would seem reasonable to regard this as adequate confirmation of the postulated pathway, in view of the considerable number of fungal phenols now known to conform to this mechanism. A corresponding assumption was made by Birch, Ryan & Smith (1958) in relation to the Kuhn–Roth degradation of labelled helminthosporin (4:5:8-trihydroxy-2-methylanthraquinone) obtained on growth of *Helminthosporium gramineum* in the presence of sodium [1-^{14}C]acetate. Confirmatory evidence of the acetate origin of this class of fungal pigments was provided independently by Gatenbeck (1958), who carried out more extensive degradations of the labelled dianthraquinone skyrin.

The results in Table 1 indicate that the incorporation of acetate into the aromatic nucleus and the isoprenoid side chain occurs to approximately the same extent. A similar finding was previously obtained by Birch, English, Massy-Westropp & Smith (1958) in an analogous study of mycophenolic acid, a product of *Penicillium brevicompactum*.

As is also the case for the other acetate-derived

phenols described thus far, the sequence of condensation of acetate units required to form the norxanthoherquein skeleton is unknown. Although, as pointed out by Barton *et al.* (1959), this ring system could arise by suitable folding of the linear poly-β-oxo acid (IV), there is at present no evidence which favours this precursor over the alternative of a branched-chain keto acid, as has been proposed for several other C_{14} fungal phenols (Thomas, 1961). The unusual nature of the C_5 side chain was discussed by Barton *et al.* (1959), who noted the existence of an identical side chain in the naphthaquinone dunnione (Price & Robinson, 1939). In both compounds the aromatic nucleus is linked to the tertiary carbon atom of the parent mevalonate unit, the original hydroxymethyl substituent of which is reduced to a methyl group. As regards the alternative structures (I) or (II) for atrovenetin, the mode of attachment of the C_5 side chain in the latter would be biogenetically preferable, as it is in accord with the normal head-to-tail linkage of acetate units. Furthermore, (II) also contains more β-orientated oxygen substituents, which is a characteristic of compounds formed in this manner.

In view of the existence of a common carbon skeleton in herqueinone, norherqueinone and atrovenetin, it is reasonable to assume that these fungal perinaphthenones arise through the same mechanism involving acetate. However, this may not be the only means of biosynthesis of this tricyclic-ring system. Thus the aglycone of the plant colouring matter haemocorin (XIV) (Cooke, Johnson & Segal, 1958), which is at present the only other known naturally-occurring perinaphthenone, contains two sets of *ortho*-orientated oxygen substituents, which is unusual among acetate-derived phenols. A more attractive biosynthetic scheme for this product would appear to involve simply the ring closure of an intermediate (XII) related to the plant substance *p*-hydroxycinnamoylferuloylmethane (XIII), isolated from the rhizome of *Curcuma longa* (turmeric) by Srinivasan (1953).

The required intermediate (XII) is postulated as arising from two C_6–C_3 units (cinnamic acid and 3:4-dihydroxyphenylpyruvic acid), which substitute into the methyl group of an acetate unit before decarboxylation. Both of these C_9 compounds would be expected to originate through shikimic acid and prephenic acid by analogy with known pathways of aromatic amino acid formation.

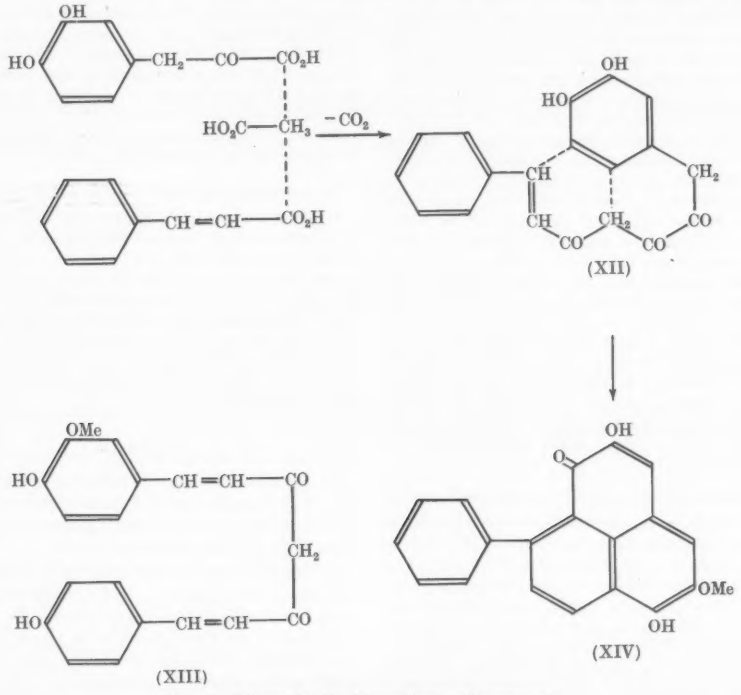

Fig. 4. Scheme for the biosynthesis of haemocorin.

On this basis, the known plant and fungal perinaphthenones would be biogenetically unrelated, despite their structural similarity.

EXPERIMENTAL

All melting points are uncorrected.

Culture and medium. The culture of *Penicillium herquei* used in these studies was the London School of Hygiene and Tropical Medicine strain no. P219. It was grown in surface culture on Raulin–Thom solution as described by Galarraga *et al.* (1955).

Radioactive assay. Samples mounted on 1·5 cm.-diam. planchets at infinite thickness were assayed for ^{14}C content with a thin end-window Geiger–Müller counter, as described by Chaplen & Thomas (1960).

Production of labelled norherqueinone. Three 1 l. flasks, each containing sterile Raulin–Thom medium (350 ml.), were inoculated with a mycelial suspension of *P. herquei* and incubated at 24° in the dark. After 13 days, 3·2 mg. of $CH_3 \cdot {}^{14}CO_2Na$ (200 μc) was added in sterile aqueous solution (5 ml.) to each flask, so as to pass directly under the mycelial pad.

Growth was continued for a further 7 days and the cultures were harvested after a total incubation period of 20 days. The metabolism solution (pH 5·9) was orange–brown; the mycelium was deeply folded and bright brownish red on the underside. The mycelia from the three flasks were combined, washed with water and dried (yield 13·33 g.).

A simplified extraction procedure was used, consisting of direct continuous extraction (90 hr.) with ether in a Soxhlet apparatus. An intense reddish-brown crystalline precipitate separated from the dark-green ether extract. This was collected by filtration and proved to be almost pure norherqueinone (0·547 g.). It was found to be highly active (19·6 μc/g.), corresponding to a minimum incorporation of approx. 1·8% of the original labelled acetate; no attempt was made to extract further product from the mother liquors.

The labelled norherqueinone (0·540 g.) was mixed with inactive pure product (2·749 g.) and recrystallized from boiling acetic acid to yield very small red needles, m.p. 279° (decomp.), of specific activity 3·31 μc/g. (1186 μc/mole). The inactive norherqueinone used for dilution purposes was obtained in a separate production experiment with 44 flasks (yield, 6·00 g.).

Addition of 9·8 mg. of DL-[2-^{14}C]mevalonic acid lactone (50 μc) to a single flask under precisely parallel conditions yielded norherqueinone (143 mg.), whose specific activity (2·64 μc/g.) corresponded to an incorporation of 0·75%. This was diluted with inactive product (0·876 g.) as described above to give recrystallized norherqueinone of activity 116 μc/mole.

Degradation of acetate-derived norherqueinone

Acid hydrolysis of norherqueinone. Norherqueinone (0·493 g.) was suspended in a mixture of 2N-H_2SO_4 (75 ml.) and acetic acid (75 ml.) and gently refluxed in a stream of N_2 for 12 hr. The effluent gases were passed through bubblers containing Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in 2N-HCl), giving rise to a yellow precipitate consisting of the 2:4-dinitrophenylhydrazone of methyl *iso*propyl ketone. This was collected by filtration (0·219 g.) and crystallized from ethanol, giving orange needles, m.p. 123° (activity 276 μc/mole).

The residue remaining after acid hydrolysis was orange, compared with the dark red of the starting product. This was filtered off, washed and dried (0·353 g.) and purified by vacuum sublimation at 270°, yielding a yellow–orange crystalline sublimate of norxanthoherquein, m.p. > 360° (activity, 908 μc/mole).

In a further experiment, norherqueinone (2·20 g.) was hydrolysed with a mixture of conc. H_2SO_4 (60 ml.), acetic acid (60 ml.) and water (120 ml.). The resulting methyl *iso*propyl ketone was retained in the mixture with the aid of an efficient reflux condenser. After 18 hr., the cooled solution was treated with sodium acetate (40 g.) in water (1 l.), thereby precipitating the norxanthoherquein, recovery and purification of which was carried out as before. The methyl *iso*propyl ketone fraction was collected by steam-distillation and oxidized directly with an alkaline solution containing a mixture of I_2 (10 g.) and KI (20 g.). A precipitate of iodoform rapidly formed, which was separated by filtration (0·60 g.) and crystallized from ethanol. This was then oxidized with chromic acid solution under van Slyke–Folch conditions and the resulting CO_2 was assayed as $BaCO_3$ (300 mg. of iodoform gave 141 mg. of $BaCO_3$, 128 μc/mole).

An attempt to recover *iso*butyric acid from the alkaline iodine oxidation filtrate as its *p*-bromophenacyl ester was abandoned because of the low yield of crude product.

Kuhn–Roth oxidation of norxanthoherquein. Norxanthoherquein (0·750 g.) was refluxed with a mixture of 5N-chromic acid (250 ml.) and conc. H_2SO_4 (75 ml.) for 3 hr. in a stream of N_2 and the effluent CO_2 was trapped as $BaCO_3$ (activity 64 μc/mole). The residual acetic acid was recovered by steam-distillation and neutralized with 0·1N-LiOH (yield, 88% theory for 1 mol.equiv.). This was partially converted into *p*-bromophenacyl acetate, m.p. 84·5–85·5° (activity, 129 μc/mole), and the remainder pyrolysed as the dry lithium salt to give acetone, which was subsequently converted into iodoform as described by Thomas (1961). The iodoform, after oxidation to $BaCO_3$, was found to be inactive.

Degradation of mevalonate-derived norherqueinone

Acid hydrolysis of norherqueinone. Norherqueinone (0·500 g.) was hydrolysed with a mixture of 2N-H_2SO_4 (75 ml.) and acetic acid (75 ml.) and the resulting volatile methyl *iso*propyl ketone was removed in a stream of N_2 and precipitated as its 2:4-dinitrophenylhydrazone as described above. This product was filtered off (0·175 g.) and purified by crystallization from ethanol (activity 114 μc/mole).

The residual norxanthoherquein was recovered (0·256 g.) and, after purification by sublimation in high vacuum, was found to be devoid of activity.

SUMMARY

1. Possible alternative pathways of biosynthesis of the fungal perinaphthenones atrovenetin ($C_{19}H_{18}O_6$), herqueinone ($C_{20}H_{20}O_7$) and norherqueinone ($C_{19}H_{18}O_7$) are discussed.

2. *Penicillium herquei* was found to incorporate sodium [1-^{14}C]acetate and DL-[2-^{14}C]mevalonic acid lactone into norherqueinone, in a manner consistent with the derivation of the perinaphthenone nucleus from acetate units and the C_5 side chain from acetate or mevalonate.

3. A scheme is proposed for the biosynthesis of

the aglycone $C_{20}H_{14}O_4$ of the plant pigment haemocorin, the only other known perinaphthenone from natural sources. This requires the coupling of one acetate and two shikimic acid-derived C_6–C_3 units and involves an intermediate related to the plant product *p*-hydroxycinnamoylferuloylmethane ($C_{20}H_{18}O_5$), which then undergoes cyclization.

The loan of tracer equipment and the purchase of ^{14}C-labelled compounds by Imperial Chemical Industries Ltd. is gratefully acknowledged. This investigation was carried out during the tenure of a Wellcome Research Fellowship.

I wish to thank Professor D. H. R. Barton, F.R.S., for his helpful comments on the chemistry of atrovenetin and herqueinone and Professor J. H. Birkinshaw for his encouragement in this investigation.

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Equilibrium Constant of Phosphoryl Transfer from Adenosine Triphosphate to Galactose in the Presence of Galactokinase

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(*Received* 8 *July* 1960)

The free energy of hydrolysis of adenosine triphosphate has been calculated as the sum of the free energies of phosphoryl transfer from this compound to $C_{(6)}$ of D-glucose and from $(\alpha+\beta)$-D-glucose 6-phosphate to water (Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957*a*, *b*; Robbins & Boyer, 1957). The equilibrium constant of the first reaction, catalysed by hexokinase, is so large that accurate estimation of the minor components of the equilibrium mixture is difficult. Colowick & Sutherland (1942) found the equilibrium constant of the phosphoglucomutase reaction,

α-D-glucose 1-phosphate
⇅
$(\alpha+\beta)$-D-glucose 6-phosphate

to be 17 at pH 7·5. An α-hexose 1-phosphate thus has a more negative free energy of hydrolysis than the corresponding hexose 6-phosphate, and the equilibrium constant of the reaction

$$\text{ATP} + \text{hexose} \rightleftharpoons \text{ADP} + \alpha\text{-hexose 1-phosphate}$$

is correspondingly smaller than that of the hexokinase reaction. Galactokinase catalyses formation of α-D-galactose 1-phosphate from adenosine triphosphate and D-galactose (Trucco, Caputto, Leloir & Mittelman, 1948; Wilkinson, 1949). It seemed probable that at pH 7·0 and 25°, in the presence of more than 10 mM-Mg^{2+}, the equilibrium constant of the galactokinase reaction would be more than 10 and less than 100.

Addition of adenylate kinase to a solution containing adenosine diphosphate and adenosine triphosphate catalyses formation of adenosine monophosphate, and ensures that

$$(\text{ADP})^2/(\text{AMP})(\text{ATP})$$

is about 2–3 at pH 7·0 and 25° in the presence of more than 10 mM-Mg^{2+} (cf. Kalckar, 1943; Eggleston & Hems, 1952; Bowen & Kerwin, 1954). By selection of a suitable ratio of total adenine derivative to total galactose derivative, the ratio of triphosphate to diphosphate may be kept in a convenient range through the coupled action of galactokinase and adenylate kinase.

In the work described here, mixtures of adenosine triphosphate and D-galactose or adenosine diphosphate and α-D-galactose 1-phosphate have been brought to equilibrium with this pair of enzymes and the equilibrium constant has been measured. The value so obtained has been used in calculation of the free energy of hydrolysis of adenosine triphosphate (Atkinson, Johnson & Morton, 1959). Combination of the equilibrium constants with published values has afforded a consistent set of free energies of hydrolysis of many phosphorylated metabolites (Atkinson & Morton, 1960 and unpublished results).

MATERIALS

Chemicals

Perchloric acid, trichloroacetic acid, formic acid and citric acid were A.R. grade (British Drug Houses Ltd.). $MgCl_2$ (A.R.; British Drug Houses Ltd.) was standardized by potentiometric determination of chloride. Diethyl ether was washed with 0·1N-NaOH, distilled and freed of peroxides with activated alumina.

D-Galactose. D-Galactose (Thomas Kerfoot Ltd., Vale of Bardsley, Lancs.) was dried to constant weight at 90° and had $[\alpha]_D^{20}$ +80·4±0·5° in water after 24 hr. (*c*, 2·0) (Found: C, 39·7; H, 6·7; O, 53·3. Calc. for $C_6H_{12}O_6$: C, 40·0; H, 6·7; O, 53·3). A single component was detected with $AgNO_3$ and alkali after chromatography in ethyl acetate–pyridine–water (2:1:1, by vol.). Analysis with hexokinase and glucose 6-phosphate dehydrogenase showed the presence of 0·8% of glucose.

α-D-Galactose 1-phosphate. (i) Material used in equilibrium studies was prepared from D-galactose (Hansen, Rutter & Krichevsky, 1955). After isolation of the potassium salt as described for potassium glucose 1-phosphate (Krahl & Cori, 1949) the ester was further purified by elution from Dowex-2 (Cl^- form) with a gradient of NaCl in HCl (Isselbacher, 1958). Chromatography in propan-2-ol–aq. 10N-NH_3 soln. (5:3, v/v) showed the presence of about 10% of orthophosphate, the only contaminating phosphate. A solution of the most concentrated eluate fractions had $[\alpha]_D^{20}$ +0·43±0·01° in a 10 cm. tube. After hydrolysis at 100° for 10 min. in 0·5N-perchloric acid it contained orthophosphate corresponding to 11·3 μmoles/ml. of original solution; the galactose content after hydrolysis in N-H_2SO_4 corresponded to 10·3 μmoles/ml. Calculated from galactose content, $[M]_D^{20}$ was +423±9°; from Wilkinson's (1949) results, $[M]_{5461}^{20}$ was calculated to be +435° for the barium salt of α-D-galactose 1-phosphate prepared enzymically. Kosterlitz (1943) reported the same rotation for ester prepared by a non-enzymic method.

(ii) The same phosphate ester was isolated from galactokinase equilibrium mixtures. Protein was removed by addition of 0·05 vol. of perchloric acid (72%, v/v) and filtration at 0°. The filtrate was brought to pH 8·6 with 10N-KOH and centrifuged. Barium acetate (5%, w/v; 12 ml.) was added to 110 ml. of the supernatant, the pH was adjusted to 9·0 with saturated $Ba(OH)_2$ and 100 ml. of ethanol was added. The barium α-D-galactose 1-phosphate was purified as in (i) above, and showed identical behaviour on chromatography. A sample which had $[\alpha]_D^{20}$ +0·13±0·01° was hydrolysed as in (i) and then contained galactose and orthophosphate corresponding to 2·6 and 3·2 mM in the unhydrolysed sample. Chromatography as in (i) showed the presence of free orthophosphate in the ester. From the galactose ester content, $[M]_D^{20}$ was +500±40°. After removal of ionic material with Dowex-50 (H^+ form) and Dowex-2 (acetate form) the sugar liberated by acid hydrolysis was chromatographed in ethyl acetate–pyridine–water (2:1:1, by vol.). The only reducing component detected coincided with authentic galactose.

Adenosine 5′-phosphate. On electrophoresis at pH 4·5 the sodium salt (Sigma Chemical Co.) gave a single component which absorbed at 254 mμ and accounted for 97% of the total absorption at 259 mμ. The adenosine 5′-phosphate (AMP) content by enzymic analysis (see Methods and Results section) was 97% of the total material absorbing at 259 mμ.

Adenosine diphosphate. The sodium salt (Sigma Chemical Co.) contained 90% of adenosine diphosphate (ADP) and 10% of adenosine triphosphate (ATP) by electrophoretic analysis. By enzymic analysis 89% of the total absorption at 259 mμ was due to ADP.

Adenosine triphosphate. The sodium salt (Sigma Chemical Co.) was used. A single component, detected by electrophoresis, accounted for 98% of the material absorbing at 259 mμ. Enzymic analyses for total reactive phosphoryl groups (~P; Slater, 1953) accounted for 99±1% of the total absorption.

Reduced diphosphopyridine nucleotide. This (reduced DPN) was prepared from oxidized DPN (98%; Sigma Chemical Co.) by reduction with ethanol and alcohol dehydrogenase (Rafter & Colowick, 1955). For use in estimation of ATP and ADP the alcohol dehydrogenase was inactivated by pouring the reaction mixture through a long-stemmed funnel into a tube preheated in boiling water. Within 2 min. the ethanol started to boil, and after a further 3 min. the solution was cooled and stored at −15°. Material prepared in this way contained traces of AMP. Reduced DPN for estimation of AMP was purified as its barium salt and freed of barium with K_2SO_4. No AMP was detected in this material.

Phosphoenolpyruvate. The barium silver salt (Sigma Chemical Co.; 100 mg.) was dissolved in 2·5 ml. of 0·2N-HNO_3 at 0°. After addition of 2N-H_2SO_4 (0·23 ml.) and removal of $BaSO_4$, AgCl was precipitated with 0·25 ml. of N-HCl and removed by centrifuging and filtration. After adjustment to pH 7 with 0·3 ml. of 2N-KOH the solution (about 3 ml.) contained 60 mM-phosphoenolpyruvate and 0·2 mM-pyruvate (estimated with lactate dehydrogenase).

Enzyme preparation

Galactokinase. This was prepared from yeast (*Saccharomyces fragilis*) adapted to galactose, which was generously supplied by the Sigma Chemical Co. The procedure described by Leloir & Trucco (1955) was modified by omission of the bentonite adsorption step. The enzyme was dialysed for

20 hr. against 0·01 M-cysteine at pH 7·0 and was freed of uridine diphosphate galactose 4-epimerase by heating for 2 min. at 50° (Maxwell, 1957). The solution contained 80 mg. of protein/ml. and catalysed phosphorylation of 0·25 μmole of galactose/min./mg. of protein in a system containing 10 mM-$MgCl_2$, 10 mM-ATP and 8 mM-galactose at 25° and pH 7·0 (assayed by titration with NaOH).

The enzyme was transported from St Louis to Adelaide in closed tubes in solid CO_2 and was stored under similar conditions. It retained its activity for over 12 months.

The enzyme contained adenylate kinase and hexokinase and traces of adenylate deaminase and phosphatase.

Adenylate kinase. This was prepared from rabbit muscle as described by Colowick (1955).

Lactate dehydrogenase. The crystalline preparation from muscle (Sigma Chemical Co.) contained enough pyruvate kinase for estimation of ADP and related compounds (see Methods and Results section).

Muscle enzymes for assay of phosphoryl donors. The following modification of the procedure described by Slater (1953) was used. The alkaline extract of muscle was brought to 30% saturation at pH 7·8 with $(NH_4)_2SO_4$ and aq. NH_3 soln. and filtered through Hyflo Supercel. The filtrate was brought to 70% saturation at pH 7·8 and filtered through fluted Whatman no. 1 paper overnight at 3°. The precipitate on the paper was washed with 70% saturated $(NH_4)_2SO_4$ at pH 7·8. When well drained, the enzyme was scraped from the paper and stored in tubes in solid CO_2; the paste retained activity for at least 12 months. For assays, fresh dilutions were prepared in 0·2 M-glycylglycine buffer at pH 7·6 and kept at 0° for not more than 12 hr. This preparation contained the following enzymes: phosphoglucomutase, phosphohexose isomerase, fructose 6-phosphokinase, aldolase, phosphotriose isomerase, glycerol phosphate dehydrogenase, adenylate kinase, and nucleoside diphosphate kinase. At the dilutions used in enzymic assays (see Methods and Results section), the material had little absorption at 340 mμ and did not oxidize reduced DPN.

Yeast alcohol dehydrogenase. The crystalline enzyme was obtained from Sigma Chemical Co.

Intestinal alkaline phosphatase. This was prepared from calf mucosa and purified as far as step 6 in the procedure as described by Morton (1954). Material which had been stored for some years was dissolved in 0·05 M-glycine, pH 9·5 and kept overnight at 2°. Insoluble material was removed by centrifuging.

METHODS AND RESULTS

Paper for chromatography and electrophoresis. Whatman no. 3 paper was washed as described by Connell, Dixon & Hanes (1955) except that treatment with calcium acetate was omitted. This was generously provided by Dr K. Rowan.

Spectrophotometry. Extinctions were measured in an Optica CF4 grating spectrophotometer calibrated for extinction with filters supplied by the National Standards Laboratories, Sydney. Wavelengths were calibrated against known lines of the mercury emission spectrum.

Electrophoretic determination of nucleotides. The following procedures were carried out in a cold room at about 3°. Samples of 20 or 25 μl. were applied as bands extending to within 2 mm. of the edges of strips (25 mm. × 300 mm.) of washed paper. The papers were dipped into 0·04 M-sodium citrate, pH 4·4–4·7, to within 2 cm. of the sample zone. Excess of buffer was removed by blotting with filter paper, and the strips were hung vertically until the wet zones had joined by capillary movement. Samples were usually analysed in quadruplicate and two blank strips were prepared.

The strips were placed on the supporting frame in a horizontal electrophoresis assembly (Evans Electroselenium Ltd.) containing the same citrate buffer. Accurate levelling of papers and buffers was essential. A potential of about 400 v was applied (approx. 14 v/cm.) for 3½ hr. The papers were then removed and dried at 25° in a horizontal position. Nucleotides were located by printing on reflex document paper with light from an unfiltered low-pressure mercury lamp (Philips TUV, 15 w; maximum emission at 254 mμ). The wet prints (after developing and rinsing) were placed on a light-box, covered with clean polythene film, and matched against the electrophoresis paper strips. Nucleotide bands were marked with a 5 mm. margin beyond the apparent limit of u.v. absorption. These zones were cut out, cut into narrow strips and placed in cylindrical funnels (approx. 20 mm. × 80 mm.) with glass-wool plugs, arranged to drain into 5 ml. volumetric flasks. In subsequent experiments, glass wool was replaced by a 5 mm. bed of no. 12 Ballotini beads. Each nucleotide band was eluted with 5 × 1 ml. portions of aq. 0·05 N-NH_3 soln. during 30 min. and the combined eluate was made up to 5 ml. Zones from corresponding regions of the blank strips were similarly eluted. Extinctions of blanks and nucleotide eluates were measured at 259 and 290 mμ in 4 cm. cells against the eluting solvent. The true extinction at 259 mμ due to nucleotide was determined from the expression:

$$E_{259\,m\mu\,(sample)} - E_{290\,m\mu\,(sample)} \times E_{259\,m\mu\,(blank)}/E_{290\,m\mu\,(blank)}.$$

Nucleotide concentrations were calculated from the equation $\epsilon_{259\,m\mu} - \epsilon_{290\,m\mu} = 15{\cdot}4 \times 10^3$ (Bock, Ling, Morell & Lipton, 1956).

Enzymic determination of nucleotides

Reactive phosphoryl groups (adenosine di- and triphosphate). The oxidation of reduced DPN (decrease in $\epsilon_{340\,m\mu} = 6{\cdot}2 \times 10^3$; Horecker & Kornberg, 1948) was followed spectrophotometrically (see Slater, 1953) in the presence of excess of glucose 6-phosphate. Usually the blank, assay and control cells (1 cm.) contained glucose 6-phosphate (2 μmoles), $MgCl_2$ (2·5 μmoles), glycylglycine–NaOH buffer, pH 7·6 (62 μmoles), and 0·2 ml. of 20% (v/v) solution of muscle-enzyme paste (see Materials section) in the same glycylglycine buffer. The assay and

control cells contained enough reduced coenzyme to give an extinction of about 0·8 and the blank and test contained the unknown (ADP + 2 ATP < 0·1 μ-mole) in a final volume of 3 ml.

Attempts to determine ATP in the presence of ADP (Slater, 1953; procedure C) with commercially available hexokinase preparations gave low values, and ATP was determined by difference between (ADP + 2ATP) (see above) and (ADP) from the phosphoryl-acceptor method below. In a series of six solutions containing 1–3 μmoles of each nucleotide/ml., the sum of (ADP + 2ATP) was measured by both the electrophoretic and enzymic methods. The ratio of the results by the two methods was $1{\cdot}00 \pm 0{\cdot}03$.

Reactive phosphoryl acceptor compounds (adenosine di- and mono-phosphate). These were determined by spectrophotometric measurement of oxidation of reduced DPN, catalysed by muscle lactate dehydrogenase in the presence of pyruvate liberated from phosphoenolpyruvate by phosphoryl transfer (see Strominger, 1955):

$$\text{Phosphoryl acceptor (X)} + \text{ATP} \xrightarrow{\text{X-kinase}} \text{X-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{pyruvate phosphate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{reduced DPN} \xrightarrow{\text{lactate dehydrogenase}} \text{DPN} + \text{lactate}.$$

Blank and assay cells (1 cm.) contained glycylglycine–NaOH, pH 7·6 (250 μmoles), $MgCl_2$ (25 μmoles), muscle lactate dehydrogenase containing pyruvate kinase (0·02 ml. of a 5 % solution in 0·02 M-glycylglycine buffer, pH 7·6), phosphoenolpyruvate (about 1·2 μmoles) and enough reduced DPN in the assay cell to give an extinction of about 0·8. The total volume was 3 ml. As shown in Fig. 1, there was no oxidation of reduced DPN before addition of phosphoryl acceptor. Equal volumes (usually 0·05 ml.) of a sample containing phosphoryl acceptor (ADP + 2AMP < 0·3 μmole) were added to each cell. The initial decrease in extinction corresponding to phosphorylation of ADP was complete within 5 min. (Fig. 1). On addition of adenylate kinase (0·02 ml. of a 5 % solution; see Materials section) there was a further decrease in extinction (Fig. 1) corresponding to oxidation of 2 equiv. of reduced DPN for 1 equiv. of AMP:

$$\text{ATP} + \text{AMP} \xrightarrow{\text{adenylate kinase}} 2\text{ADP}.$$

Some samples of lactate dehydrogenase–pyruvate kinase contained traces of adenylate kinase which prevented the accurate determination of ADP in a mixture containing AMP. It was found that lactate dehydrogenase diluted in the phosphoenolpyruvate–$MgCl_2$–glycylglycine solution (see above) lost the adenylate kinase activity on storage at 0° for about 14 hr.

The amounts of ADP and AMP were calculated from the change in $E_{340\,m\mu}$ (see Fig. 1). In a series of seven determinations, the ratio of values determined enzymically and electrophoretically for ADP was $1{\cdot}00 \pm 0{\cdot}03$ and for AMP was $0{\cdot}99 \pm 0{\cdot}06$.

Where suitable kinase preparations are available the enzymic procedure as described here is useful for determination of other phosphoryl acceptors. If the reaction mixture does not contain ADP or ATP, about 1 μmole of the latter should be added to initiate phosphorylation of the acceptor (< 0·3 μ-mole in this system).

After ADP and AMP had been determined as described above, addition of inosine 5′-phosphate (2 μmoles) to the assay cell caused no further decrease in extinction. Subsequent addition of muscle-enzyme preparation (0·025 ml. of a 20 % solution of paste in 0·2 M-glycylglycine buffer, pH 7·6), which contained nucleoside diphosphate kinase, resulted in a rapid oxidation of reduced DPN, corresponding to the phosphorylation of inosine 5′-phosphate. Thus the measured AMP concentration did not include the inosine 5′-phosphate, which could be separately estimated.

Estimation of galactose

Galactose was estimated colorimetrically by the arsenomolybdate method (see Nelson, 1944). In earlier experiments, protein and phosphates were precipitated with $ZnSO_4$–$Ba(OH)_2$ reagent. However, interfering compounds in the galactokinase equilibrium mixtures were not entirely removed by this procedure. In later work, samples of equilibrium mixture (0·3 ml.) which had been freed of protein by the trichloroacetic acid–ether procedure (see below) were applied to 40 mm. × 300 mm. strips of washed filter paper, and electrophoresis was carried out at 12 v/cm. for 8 hr. in 0·04 M-Na_2HPO_4 at 3°. As an internal standard, 0·025 ml. of galactose (1·00 mg./ml.) was applied to two of the four test strips and to one of the two blank strips.

The compounds which absorbed at 254 mμ were first located photographically as already described, and then galactose was located by treatment of marker strips with alkaline silver reagent. In this system the nucleotides and galactose 1-phosphate were well separated from the uncharged galactose which was then eluted as described for nucleotide estimation with five 1 ml. portions of water (final vol., 5·0 ml.).

Duplicate 2 ml. portions of the six eluates were mixed with 0·25 ml. of galactose (100 μg./ml.) to bring the galactose concentration to the most suitable region of the standard line. Alkaline copper reagent A + B (2 ml., see Nelson, 1944) was added

to each tube and to a series of standards. After thorough mixing of the contents, the tube was kept for 35 min. in boiling water and cooled for 5 min. under tap water. The contents were mixed with 2 ml. of arsenomolybdic acid reagent, and after 20 min. diluted with water to 20 ml. Extinctions (2 cm. light path) were read at 600 mμ within 2 min. of mixing. The times of initial mixing and heating were arranged so that each tube was treated in the same way. Extinction was a linear function of the amount of galactose over the range 0–90 μg./tube with an extinction range 0·1–1·0. The mean recovery of the internal standard, added before electrophoresis, was 95 %.

Other procedures

Measurement of pH. This was done at 25·0° with a glass electrode and pH meter which had been calibrated against standard phthalate and borate buffers. Small glass and calomel electrodes for use in narrow tubes were constructed by N. L. Jones, Melbourne.

Removal of protein from equilibrium mixtures. Two procedures were used. Formic acid was added to a final concn. of 10 % (v/v), protein was removed by centrifuging, and a definite volume of the supernatant was freeze-dried in a tube previously marked at the original meniscus. The dry residue was dissolved in water and made up to the initial volume. Alternatively, 1 ml. of trichloroacetic acid (50 %, v/v) was added to the equilibrium mixture (8 ml.) in a tube previously marked at the meniscus. After 2 min. at 25°, the solution was cooled to 0° and then extracted with diethyl ether (4 × 10 ml.). The aqueous solution was concentrated to about 7 ml. at 30° in a stream of N_2 and diluted to 8 ml. with water. Solutions were kept in closed tubes in solid CO_2 until analysed.

No significant difference was found between nucleotide analyses of materials freed of protein by the formic acid and trichloroacetic acid procedures. The latter procedure was used in obtaining the equilibrium concentrations reported here.

Establishment of equilibria

(*a*) *Initial reactants, adenosine triphosphate and galactose.* Into a tube with a mark at 8 ml. were added galactose (18·5 mg., 102 μmoles), ATP (44 mg., 65 μmoles), $MgCl_2$ (0·25 M, 0·8 ml.) and water (3 ml.). The solution was stirred with a stream of N_2 and kept at 25·0°, and the pH was brought to 7·0 by addition of 0·25 N-NaOH from a microsyringe. Then 0·075 ml. of galactokinase and 0·01 ml. of adenylate kinase were added. The pH was kept at 7·0 by addition of alkali to neutralize acid liberated on phosphorylation of the galactose, according to the reaction:

$$\text{ATP} + \text{D-galactose} \rightarrow \text{ADP} + \alpha\text{-D-galactose 1-phosphate} + H^+$$

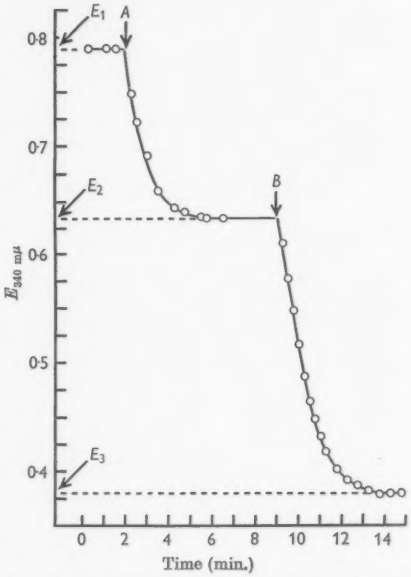

Fig. 1. Determination of ADP and AMP with coupled dehydrogenase–pyruvate kinase–adenylate kinase system. The details of the procedure are given in the Methods and Results section. At *A*, 0·05 ml. of solution containing ADP (0·065 μmole) and AMP (0·053 μmole) was added. At *B*, adenylate kinase (0·02 ml.) was added. Extinctions are corrected for dilution to the final volume, 2·6 ml. The concentration of ADP (mM in cell) was calculated from $(E_2 - E_1)/6{\cdot}2$, and the concentration of AMP (mM in cell) from $(E_2 - E_3)/(6{\cdot}2 \times 2)$.

Three successive 0·05 ml. portions of galactokinase were then added at approx. 2 hr. intervals and, when no further pH change could be detected, 0·01 ml. of adenylate kinase was added, and the solution was diluted with water to 8·0 ml. After a further 20 min at pH 7·00 and 25°, the solution was freed of protein with trichloroacetic acid (see above). Galactose, ATP, ADP and AMP were estimated as already described. Some inosine 5′-phosphate, adenosine and inosine were formed as a result of adenylate deaminase and phosphatase activities. These compounds were well separated from the adenyl nucleotides by electrophoresis and caused no interference with enzymic assays. Galactose 1-phosphate was estimated by difference between the initial and final concentrations of galactose. In one experiment (see Table 2) the solution was treated with phosphatase, which liberated all the galactose initially present. There was no conversion of galactose into compounds other than α-D-galactose

1-phosphate as demonstrated by enzymic assays for glucose phosphates (Slater, 1953) and by characterization of the phosphorylated sugar as α-D-galactose 1-phosphate (see Materials section). Results are given in Tables 1 and 2.

(*b*) *Initial reactants, adenosine diphosphate and galactose 1-phosphate.* Into a tube were put ADP (36 mg., 68 μmoles), α-D-galactose 1-phosphate (51·3 μmoles in 5 ml.), and $MgCl_2$ (0·25 M, 0·8 ml.). As previously described, the pH was brought to 7·0 and after addition of galactokinase and adenylate kinase was kept at 7·0 by addition of 0·25 N-HCl and 0·25 N-NaOH. When there was no further change of pH, water was added to 8 ml. and after a further 20 min. at pH 7·0 the solution was freed of protein with trichloroacetic acid and analysed as described for (*a*) above. Results are given in Table 1.

DISCUSSION

The free-energy change of phosphoryl transfer from ATP to the α-anomer of D-galactose is so small at pH 7·0 that all the reactants at equilibrium may be measured by specific methods. With the precautions already described, spectrophotometry after electrophoretic purification has proved a satisfactory method for the analysis of AMP, ADP and ATP. The standard error in determination of ADP and ATP by this method was 1–3 % of the mean value for 1–5 mM-solutions; a similar precision was possible in the determination of AMP, but longer runs were needed for purification of this compound, and in Table 1 equilibrium constants were calculated from AMP concentrations determined by the enzymic procedure.

Table 1. *Equilibrium constant of the galactokinase reaction*

For experimental conditions see text; the concentration of Mg^{2+} ions was 25 mM.

	Equilibrium concentrations (mM)	
	From (α + β)-D-galactose and ATP	From α-D-galactose 1-phosphate and ADP
ATP (by electrophoresis)*	1·06 ± 0·02 (4)	1·51 ± 0·04 (4)
ADP (by electrophoresis)*	2·78 ± 0·03 (4)	2·49 ± 0·01 (4)
ADP (by enzymic assay)	2·81 ± 0·08 (4)	2·47 ± 0·02 (3)
2ATP + ADP (by enzymic assay)	4·75 ± 0·06 (4)	5·30 ± 0·07 (4)
2ATP + ADP (by electrophoresis)	4·90 ± 0·04 (4)	5·51 ± 0·08 (4)
AMP (by electrophoresis)	2·18 ± 0·12 (4)	1·12 ± 0·07 (4)
AMP (by enzymic assay)*	2·05 ± 0·03 (4)	1·17 ± 0·02 (3)
D-Galactose	1·2 ± 0·1 (8)	0·37 ± 0·04 (8)
α-D-Galactose 1-phosphate (by difference; see text)	11·6 ± 0·1	6·1 ± 0·1
†K_{Gal}	25 ± 3	27 ± 3
‡$\Delta G'$ (kcal./mole)	−1·9 ± 0·1	−1·9 ± 0·1
$[ADP]^2/[AMP][ATP]$	3·6	3·5

* Value used in calculation of equilibrium constants.

† $K_{Gal} = \dfrac{[ADP][\alpha\text{-D-galactose 1-phosphate}]}{[ATP]\,[(\alpha+\beta)\text{-D-galactose}]}$.

‡ $\Delta G' = -RT \ln K_{Gal}$.

Table 2. *Preliminary galactokinase equilibria*

These results were obtained with 10 mM-Mg^{2+} at pH 7·0 and 25·0°; for experimental details, see text.

Equilibrium concentrations (mM)							
ATP	ADP	AMP	Galactose	Galactose 1-phosphate	K_{Gal}†	$\Delta G'$ (kcal./mole)†	$\dfrac{[ADP]^2}{[ATP][AMP]}$
0·94	2·35	2·83	1·79	11·0	15	−1·6	2·1
0·91	2·40	2·91	1·67	11·1	18	−1·7	2·2
1·11	2·39	2·43	0·82	10·4	27	−1·9	2·1
1·51	2·45	1·83	0·40	9·2	37	−2·1	2·2
1·65	2·50	1·97	0·39	9·2	36	−2·1	1·9
3·3	3·5	2·1	0·47*	7·6	17	−1·7	1·8
3·92	2·36	0·66	0·11	3·9	21	−1·8	2·2
				Mean	24 ± 9	−1·9 ± 0·2	2·1 ± 0·2

* After hydrolysis with phosphatase 8·4 ± 0·8 (for details see text).
† See Table 1.

Phosphoryl transfer from phosphoenolpyruvate to ADP and enzymic reduction of the pyruvate liberated afforded confirmation of the electrophoretic assay of the diphosphate; concentrations measured by the two methods did not differ by more than 1% (Table 1). Extension of this assay by coupling with adenylate kinase (see Methods and Results section and Fig. 1) provided a sensitive method for estimation of AMP; the standard error was about 2% of the mean value. This assay of phosphoryl acceptors is widely applicable if suitable kinases, free of adenosine triphosphatase and other phosphatases, are available. Slater's (1953) enzymic assay of phosphoryl donors (~P) was used for comparison with the electrophoretic determination of (ADP + 2ATP); differences between the two types of results were not more than 4%, and in the preliminary experiments (Table 2) were less than 3%.

Estimation of galactose was the greatest source of error in these experiments. In Table 1, where results shown were obtained after electrophoretic purification, the recovery of 95% of internal standards, essentially equal in concentration to the unknown galactose, and the standard error (about 10% of the mean) indicate the probable reliability of the analysis. Although the glucose content of the original galactose was less than 1% (for details see Methods and Results section), this would have been a serious contaminant in the small proportion of residual galactose, but for the presence of hexokinase, which catalyses almost complete phosphorylation of glucose in these conditions (Vladimirov *et al.* 1957*a*; Robbins & Boyer, 1957).

After it had been shown (Table 2) that phosphatase hydrolysed all the esterified galactose to a reducing sugar, α-D-galactose 1-phosphate was estimated as the difference between the initial and final galactose concentrations. Examination of equilibrium mixtures by enzymic procedures (Slater, 1953) and of the hexose phosphate before and after hydrolysis (see Methods and Results section) gave no evidence of conversion of galactose into any compound other than α-D-galactose 1-phosphate. As the ester was the major component at equilibrium its subsequent estimation by difference introduced a relatively small error.

Although the hexose analyses in the preliminary experiments with 10 mM-$MgCl_2$ (Table 2) were less satisfactory than in later experiments (Table 1), the standard error of individual values of K_{Gal} in Table 2 is probably less than 20%. The variation observed in the series of experiments shown in Table 2 (K_{Gal}, 24 ± 9) is probably due to competition for Mg^{2+} ions between the phosphorylated reactants resulting in different extents of magnesium complex formation. At the higher magnesium chloride concentration (25 mM) used in the later experiments (Table 1) there is a considerable excess of Mg^{2+} ions with respect to phosphorylated reactants (cf. Robbins & Boyer, 1957; Burton, 1959) and these differences are apparently avoided. Thus in these conditions K_{Gal} was 25 ± 3 with galactose and ATP as the initial reactants, and 27 ± 3 with α-D-galactose 1-phosphate and ADP, despite considerable differences in the proportions of reactants. The effect of the concentration of Mg^{2+} ions on the equilibrium constant of the adenylate kinase reaction is also evident from Tables 1 and 2.

SUMMARY

1. Electrophoretic and enzymic methods are described for specific estimation of compounds present in the equilibria:

$$ATP + (\alpha+\beta)\text{-D-galactose} \rightleftharpoons ADP + \alpha\text{-D-galactose 1-phosphate},$$

and

$$2ADP \rightleftharpoons AMP + ATP,$$

which are catalysed by galactokinase and adenylate kinase respectively.

2. A general spectrophotometric estimation of the substrates of kinases is described.

3. The equilibrium constant of the galactokinase reaction [ADP][α-D-galactose 1-phosphate]/[ATP][(α + β)-D-galactose] was 26 at pH 7·00 and 25° in the presence of 25 mM-Mg^{2+}.

4. The equilibrium constant of the adenylate kinase reaction $[ADP]^2/[AMP][ATP]$ at pH 7·00 and 25° was 2·1 with 10 mM-Mg^{2+} and 3·6 with 25 mM-Mg^{2+}.

We wish to thank Mr A. C. Jennings for advice and assistance in the determination of galactose, and Mr H. R. Lovelock for preparation of the galactose phosphate. The technical assistance of Miss Eleanor Johnson and Mr M. P. Radic is also gratefully acknowledged. Carbon, hydrogen and oxygen were determined by the Australian Micro-Analytical Service. One of us (R.M.B.) was supported by a grant, no. B-1575, from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

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Haemolysins in Venoms of Australian Snakes

OBSERVATIONS ON THE HAEMOLYSINS OF THE VENOMS OF SOME AUSTRALIAN SNAKES AND THE SEPARATION OF PHOSPHOLIPASE A FROM THE VENOM OF *PSEUDECHIS PORPHYRIACUS*

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(*Received* 27 *June* 1960)

A supply of phospholipase A which was free from other enzymes was required during a study of the antagonism between staphylococcal toxin and certain venoms (North & Doery, 1958). Phospholipase A had already been separated from venoms of the honey-bee (Habermann & Neumann, 1957) and *Crotalus terrificus terrificus* (Habermann, 1957). However, for our purpose venoms of Australian species of snakes were considered as a source of this enzyme. The haemolytic activities of a number of these venoms were first studied by Kellaway & Williams (1933), Holden (1934, 1935), Feldberg & Kellaway (1937, 1938) and Trethewie (1939). Holden (1934) showed that washed red cells of the rabbit were haemolysed by solutions of these venoms at 37°. He observed an increase of activity on the addition of lecithin. It was generally agreed at that time, on chemical and pharmacological evidence, that the haemolytic activities of the venoms of the Australian snakes studied were dependent on the formation of lysolecithin by phospholipase A present in the venoms (Feldberg & Kellaway, 1938; Trethewie, 1939).

It is known that there are two types of venom haemolysins, 'direct' and 'indirect' (Holden, 1935; Neumann & Habermann, 1952; Habermann, 1954). The direct haemolysin lyses washed red cells of certain species readily without added lecithin, whereas the indirect haemolysin lyses such cells only feebly, but haemolysis is markedly enhanced by the addition of lecithin, which is converted into the haemolytic agent, lysolecithin, by the phospholipase A.

Both types of haemolysins have been found among the common venomous species of Australian snakes, the venom of *Pseudechis porphyriacus* being particularly rich in both. This venom was readily available and was chosen as a source of phospholipase A. In this paper the concentrations of both types of haemolysins which have been found in venoms of some Australian snakes will be recorded. Certain other venoms considered to be strongly haemolytic were also included in this study. The separation of phospholipase A from the venom of *P. porphyriacus* will be described and discussed in relation to the occurrence of this enzyme in some other venoms.

MATERIALS AND METHODS

Dried venoms. The venoms of the Australian snakes were either purchased from E. Worrell, Wyoming, Gosford North, N.S.W., Australia, or collected at these Laboratories and prepared as already described (Doery, 1958). Other venoms were obtained from Ross Allen's Reptile Institute, Florida, U.S.A., and the Medical Research Institute, Johannesburg, South Africa.

Fractions of the venom of Notechis scutatus. These were prepared in the course of separating the neurotoxins of this venom (Doery, 1958) when they were called fraction 1 which was the haemolysin-rich fraction, fraction 1+ and fraction 2.

Crude lecithin. This was prepared from egg yolk by the method described by Saunders (1957).

Preparation of resin Amberlite IRC-50 (XE-64). The resin was prepared for chromatography as described by Doery (1958).

Paper electrophoresis and elution. A hanging-strip type of cell was used (Spinco Model R) with 3 cm. wide strips of Whatman 3 MM paper. The apparatus was operated at constant current (5–25 mA/cell) and at 0–4°. Samples (500 μg.) were examined as 5% (w/v) solutions and applied as a band across the centre of each strip. Dextran was used as an indicator of the extent of electro-osmosis. The strips were stained and eluted as described by Doery (1958).

Preparation of red cells. Blood cells (human) were stored in Rous & Turner (1916) suspending fluid, and washed three times in 0·9% sodium chloride soln.

Estimation of direct haemolysin. The procedure was essentially that of Neumann & Habermann (1954), all dilutions being made in Sörensen's buffer, pH 7·0, diluted 1/5 with 0·9% sodium chloride soln. Portions (0·5 ml.) of a 2% (w/v) suspension of red cells were added to 0·5 ml. lots of venom solutions in a suitable range of dilutions, incubated at 37° for 30 min., and then held in an ice bath until centrifuged. To each supernatant, one drop of aq. 1% (w/v) potassium ferricyanide soln. was added, and the extinction at 630 mμ was measured. From the curves relating venom concentration and extinction at 630 mμ, the haemolytic activity of the venom solution was expressed as a percentage of that of the appropriate control.

Estimation of phospholipase A. The method was essentially that of Neumann & Habermann (1955), all solutions and suspensions being made in 0·9% sodium chloride soln. Portions (2·0 ml.) of 40% (v/v) suspension of egg yolk were added to 0·2 ml. lots of venom solutions covering an appropriate range of dilutions, and incubated at 37° for 2 hr. The reaction mixtures were then held in an ice bath until 0·2 ml. portions were removed. These were incubated with 4 ml. lots of a 0·6% (v/v) suspension of washed red cells (human) at 37° for 30 min. At the completion of this reaction the mixtures were held in an ice bath until they were spun in a refrigerated centrifuge at 5°. The extinctions of the supernatants were measured at 576 and 620 mμ. The difference between these gave a measure of the extent of haemolysis. From the curves relating concentration and percentage of complete haemolysis, the phospholipase A activity was expressed as a percentage of that of the appropriate control measured over a tenfold concentration range. By using the same preparations of egg-yolk substrate and red-cell suspension the mean deviation of the results was 12%, increasing to 15% for day-to-day estimations, when fresh substrate and red-cell suspensions were used. The results presented below are within these limits. In some experiments crude lecithin from egg yolk was used as the substrate.

Comparison of the haemolytic activities of venoms. For the study of the haemolytic activities of the venoms of some Australian snakes and other selected venoms, one sample of each venom only was examined. The direct haemolytic and phospholipase A activities of the sample of venom of *P. porphyriacus* served as standards of comparison and were taken as 100%. Venom solutions were examined in 50% dilution steps over the appropriate range of concentrations, and the direct haemolytic and phospholipase A activities were expressed as percentages of the activity of the venom of *P. porphyriacus* to the nearest 50%.

Estimation of cholinesterase. This was determined colorimetrically by the method of McCosker & Daniel (1959).

Estimation of the coagulating factor, hyaluronidase and 5′-nucleotidase. The methods used were those described by Doery (1958). 5′-Nucleotidase activity was examined against one substrate only, namely adenosine 5′-phosphate.

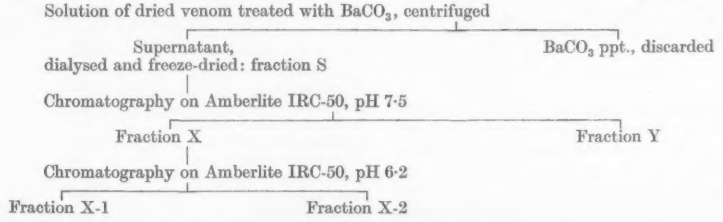
Procedures for fractionation of the phospholipase A in the venom of Pseudechis porphyriacus

These are outlined in Scheme 1.

Removal of the coagulating factor. This was carried out by adsorption on barium carbonate (Holden, 1933) as described by Doery (1958).

Ammonium acetate buffers. Aqueous solutions of ammonium acetate of the required molarity were adjusted to the selected pH with the addition of either aq. ammonia soln. or acetic acid.

Chromatography on Amberlite IRC-50 (XE-64) *at room temperature* (15–25°). The freeze-dried supernatant from the barium carbonate, fraction *S*, was examined by chromatography on this resin; 1·40 mg. were applied as a 10% (w/v) solution in 0·1 M-ammonium acetate, pH 7·5–7·8, to a


Solution of dried venom treated with $BaCO_3$, centrifuged
Supernatant, dialysed and freeze-dried: fraction S
$BaCO_3$ ppt., discarded
Chromatography on Amberlite IRC-50, pH 7·5
Fraction X
Fraction Y
Chromatography on Amberlite IRC-50, pH 6·2
Fraction X-1
Fraction X-2

Scheme 1. Fractionation procedures

column of the resin (2·5 cm. × 18 cm.) which had been previously equilibrated with this buffer. Elution with the same buffer was followed by the measurement of the extinction at 278 mμ of successive eluates. After emergence of the first protein peak and when no further protein was eluted, the eluent was changed to M-ammonium acetate, pH 7·5, and a second peak emerged. Appropriate eluates within each protein peak respectively were combined and the products freeze-dried to give fractions X and Y (Fig. 1). Fraction X from each of three such columns was combined and subjected to chromatography again on the resin Amberlite IRC-50 equilibrated with 0·1 M-ammonium acetate at pH 6·2–6·3. With this buffer as the eluting fluid two protein peaks were eluted in succession. When no further protein was eluted, the eluent was replaced by M-ammonium acetate at the same pH, but this failed to elute more than 1% of additional protein. Appropriate eluates in each peak were combined, and, after freeze-drying, two fractions X-1 and X-2 were obtained (Fig. 2).

In early experiments fraction X was subjected to chromatography with the same system, namely the resin Amberlite IRC-50 equilibrated with 0·1 M-ammonium acetate, but at a lower pH, namely 6·0. Fraction X-1 was eluted at the elution volume, as was the case at the higher pH, but the remainder of fraction X was more strongly adsorbed and eluted only by changing the eluent to M-ammonium acetate at pH 6·0. The resultant fraction had a lower phospholipase A activity and was less homogeneous than fraction X-2 as subsequently prepared.

Dialysis of venom fractions. Aqueous solutions of the various fractions were dialysed in cellophan tubing against distilled water at 4° for 16 hr. The contents of the tubing were then analysed for phospholipase A.

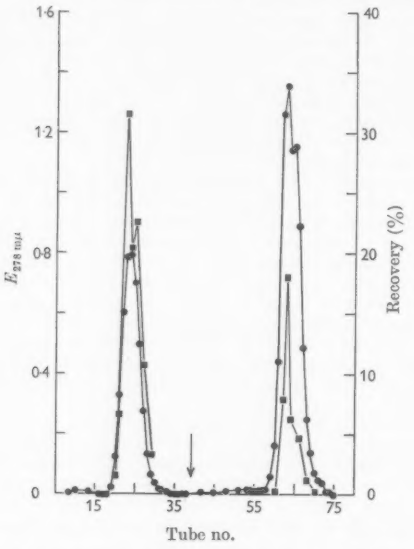


Fig. 1. Chromatography on Amberlite IRC-50 (XE-64) of 140 mg. of fraction S. Eluting buffer was 0·1 M-ammonium acetate, pH 7·7; at the arrow the eluent was changed to M-ammonium acetate, pH 7·5. ●, Extinction at 278 mμ; ■, % recovery of phospholipase A activity. Contents of tubes were pooled to provide the following fractions: tubes 20–29, fraction X; tubes 60–70, fraction Y.

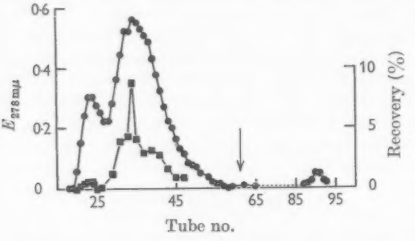


Fig. 2. Chromatography on Amberlite IRC-50 (XE-64) of 120 mg. of fraction X. Eluting buffer was 0·1 M-ammonium acetate, pH 6·2; at the arrow the eluent was changed to M-ammonium acetate, pH 6·2; ●, Extinction at 278 mμ; ■, % recovery of phospholipase A activity. Contents of tubes were pooled to provide the following fractions: tubes 20–26, fraction X-1; tubes 30–47, fraction X-2.

RESULTS

In preliminary studies it became evident that the method chosen for estimating phospholipase A was subject to considerable variation. This was decreased somewhat by holding samples at low temperatures wherever possible while sampling, as already indicated. Table 1 records the concentrations of phospholipase A and the direct haemolysin found in a number of venoms, expressed as percentages of those found in the venom of *P. porphyriacus*.

The haemolytic activity of the venom of *Notechis scutatus* was previously estimated by its weak activity as a direct haemolysin (Doery, 1958), and it was assumed to be phospholipase A because it was known to be enhanced by the addition of lecithin. Subsequent estimations of the phospholipase A activity by the method described in this

Table 1. *Haemolytic activity of venoms as percentages of that of the venom of* Pseudechis porphyriacus

Venom	Phospholipase A	Direct haemolysin
Pseudechis porphyriacus	100	100
P. australis	150	50
P. papuanus	150	100
Denisonia superba	50	50
Notechis scutatus scutatus	50	2–3
Oxyuranus scutellatus	100	<1
Acanthophis antarticus	50	<1
Demansia textilis textilis	<20	<12
Naja nigricollis	50	600
Vipera russelli	25	<10
Agkistrodon piscivoris	12	<3

paper confirmed this identification. In Table 2 the recovery of the haemolytic activity in three fractions of this venom by both methods of estimation are recorded. Fraction 1 is hereafter referred to as the phospholipase A-rich fraction of the venom of *Notechis scutatus*.

The separation of phospholipase A from the venom of *P. porphyriacus* was followed by the estimation of both this enzyme and the direct haemolysin at each step in the procedure outlined in Scheme 1. Table 3 records the recovery of the mass (dry wt. over P_2O_5 *in vacuo*), the phospholipase A and the direct haemolysin in each fraction, expressed as percentages of the original dried venom.

The barium carbonate treatment removed more than 90% of the clotting factor and no less than 98% of both the hyaluronidase and cholinesterase activities of the dried venom. However, 40, 51 and 98% of the 5′-nucleotidase, the direct haemolysin and phospholipase A activities respectively, remained in the supernatant, i.e. fraction S. The complexity of this fraction S was also indicated by paper electrophoresis.

The separation of fraction S by chromatography on the resin Amberlite IRC-50 at pH 7·7 and 7·5 into two fractions X and Y, both of which contain phospholipase A activity, is illustrated by a typical elution curve in Fig. 1. An overall recovery was obtained of 82% of the protein applied to the column. Fractions X and Y contained 29 and 49% respectively of the protein and 54 and 30% respectively of the phospholipase A applied to the column. The further separation of fraction X by chromatography at pH 6·2 into the two fractions X-1 and X-2, both of which again contained phospholipase A, is illustrated by the elution curve in Fig. 2. With an overall recovery of 81% of the protein, fractions X-1 and X-2 contained 15 and 57% respectively of the protein and 4 and 117% respectively of the phospholipase A applied to the column, the latter figure being within the experimental error inherent in the method of analysis. These fractionation procedures finally resolved the venom of *P. porphyriacus* into the three fractions X-1, X-2 and Y which contained 3, 70 and 37% respectively of the activity of the original dried venom (Table 3).

In the ultracentrifuge, X-2 [1·4% (w/v) in 0·2 M-sodium chloride] moved as a symmetrical peak in a field of 250 000g with $S_{20,w}$ of 2·3. However, the spread was greater than one might expect for a single component.

Thus fraction X-2 is a preparation of phospholipase A with seven times the activity of the crude venom on the activity:mass ratio, whereas fractions X-1 and Y have about 0·6 and 0·8 of the activity on the same basis. Figs. 3–6 show paper-electrophoresis-elution curves of these three fractions of the venom of *P. porphyriacus* together with the phospholipase A-rich fraction of the venom of *Notechis scutatus* (Doery, 1958). The latter fraction, previously shown to be inhomogeneous, was resolved into two distinct components by paper electrophoresis in veronal buffer at pH 8·6 by the procedure used here (Fig. 3). Fraction X-1 was similarly resolved and showed a paper-electrophoresis pattern approximating to that of the fraction of the venom of *Notechis scutatus* in both veronal buffer at pH 8·6 and ammonium acetate buffer at pH 6·0 (Figs. 3 and 4). Fraction X-2 more closely followed the movement

Table 2. *Recovery of haemolytic activity of the venom of* Notechis scutatus *in fractions of this venom expressed as percentages of the dried venom*

Fraction	Method of estimation: Direct haemolysin*	Method of estimation: Phospholipase A
1	71	66
1+	4	4
2	3	2

* Doery (1958).

Table 3. *Recovery of mass, phospholipase A and direct haemolysin as percentages of dried venom of* Pseudechis porphyriacus

Fraction	Mass	Phospholipase A	Direct haemolysin
Dried venom	100	100	100
Supernatant from $BaCO_3$ ppt. freeze-dried, fraction S	78	98	51
Chromatography of fraction S on Amberlite IRC-50:			
Fraction X	22	66	3
Fraction Y	45	37	23
Chromatography of fraction X on Amberlite IRC-50:			
Fraction X-1	5	3	<1
Fraction X-2	10	70	<1

of dextran in ammonium acetate buffer at pH 6·0 than in veronal buffer at pH 8·6, indicating an isoelectric point in the region of pH 6 (Figs. 5 and 6). Fraction Y, shown to be inhomogeneous under both conditions of electrophoresis, contained components which overlapped with fractions X-1 or X-2 and included the most cationic components of the venom (Figs. 5 and 6).

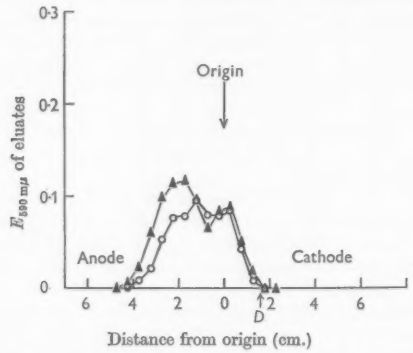


Fig. 3. Paper-electrophoresis-elution curves of venom fractions (500 μg.). ▲, Phospholipase A-rich fraction of venom of *Notechis scutatus*; ○, fraction X-1; *D*, position of dextran. Veronal buffer had *I* 0·05 and pH 8·6; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

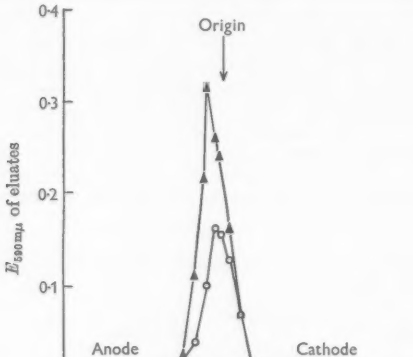


Fig. 4. Paper-electrophoresis-elution curves of venom fractions (500 μg.). ▲, Phospholipase A-rich fraction of venom of *Notechis scutatus*: ○, fraction X-1; *D*, position of dextran. The buffer was 0·2 M-ammonium acetate, pH 6·0; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

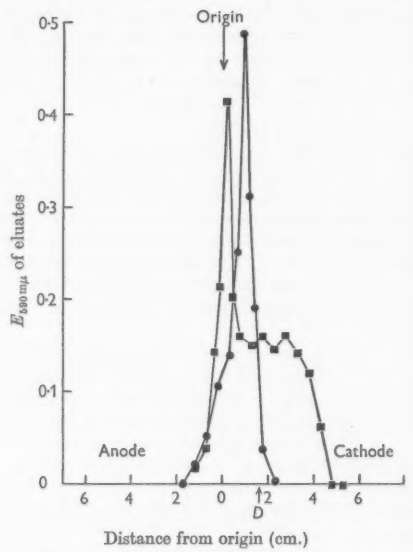


Fig. 5. Paper-electrophoresis-elution curves of venom fractions (500 μg.). ●, Fraction X-2; ■, fraction Y; *D*, position of dextran. Veronal buffer had *I* 0·05 and pH 8·6; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

All attempts to fractionate Y further have only added to the number of fractions with phospholipase A activity. In view of the possibility that the activity of fraction Y may have been due to incomplete separation from fraction X, fraction Y was re-examined by chromatography on the resin Amberlite IRC-50 at pH 6·2. However, although 30 % of the activity could then be eluted at pH 6·2 to give a preparation equal in activity to that of the crude venom on an activity : mass ratio, the paper electrophoresis pattern showed that it was still inhomogeneous and contained predominantly cationic components. Further attempts to separate the activity into a single component from fraction Y by means of precipitation with methanol at low temperatures over a range of pH values were equally unsuccessful. Phospholipase A activity was found in all portions of fraction Y which were separated.

Fraction S contained 40 % of the 5′-nucleotidase activity of the crude venom, but < 5 % of the original activity was found in each of the fractions X-1, X-2 and Y.

On dialysis against distilled water at 4° fraction X-1 lost 30 % of its activity with no detectable loss in protein. The phospholipase A-rich fraction of the venom of *Notechis scutatus* also showed a

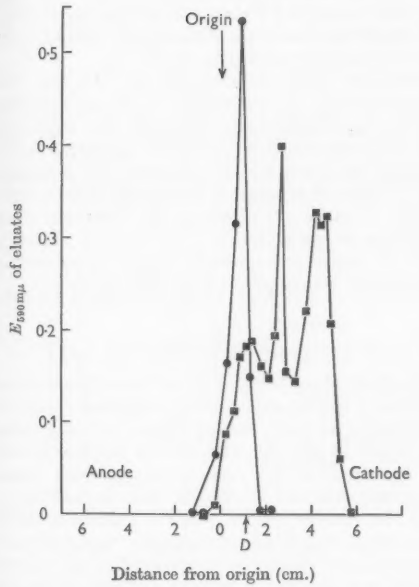


Fig. 6. Paper-electrophoresis-elution curves of venom fractions (500 μg.). ●, Fraction X-2; ■, fraction Y; *D*, position of dextran. Buffer was 0·2 M-ammonium acetate, pH 6·0; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

similar loss of activity on dialysis. Fractions X-2 and Y were dialysed without loss of activity.

A limited study of possible substrate specificity or activation was begun by comparing the activity of fractions against both crude lecithin and whole egg yolk as substrates. The ratio of the phospholipase A activity against these two substrates was compared for each of the three fractions X-1, X-2 and Y. Fraction X-2 showed a markedly lower ratio of activity against crude lecithin to activity against whole egg yolk than did either of the other two fractions. Sodium oleate when added to crude lecithin doubled the activity of fraction X-2 against this substrate, whereas no significant effect on the activities of fraction X-1 or Y could be detected.

When the distribution of the direct haemolysin is considered, Table 3 shows that, although fraction S contained about 50 % of the direct haemolytic activity of the dried venom, only approximately 50 % of this was recovered in fraction Y. Moreover, in attempts that were made to separate the various components of fraction Y we failed to find a component in which the direct haemolysin was specifically concentrated.

DISCUSSION

This study included the venom of eight of the common venomous species of Australian snakes. As found by earlier workers, phospholipase A is widely distributed. The venom of *Demansia textilis textilis* is an exception in this regard. The concentrations in which it occurs in the venoms studied are as high or higher than those found in samples of three venoms, from other than Australian species of snakes, namely *Naja nigricollis*, *Vipera russelli* and *Agkistrodon piscivoris*, which are frequently regarded as rich sources of phospholipase A.

The direct haemolysin is, however, less universally found within venoms of Australian species, the highest concentrations being found in those of the *Pseudechis* species of snakes studied. In the venoms of *Notechis scutatus scutatus*, *Oxyuranus scutellatus*, *Acanthophis antarticus* and *D. textilis textilis*, this haemolysin was either not detectable or present in a very low concentration relative to that in the venom of *P. porphyriacus*. This haemolysin is apparently less stable or possibly more strongly adsorbed to the resin than phospholipase A. This is borne out by the low overall recovery (23 % in fraction Y) which was obtained in the course of separating phospholipase A from the latter venom. We have failed to identify this activity with a single component of the venom. This activity is associated with the most electropositive fraction of both the venom studied here, *P. porphyriacus*, and that of the honey-bee (Neumann & Habermann, 1954).

Reference was made earlier to the fact that phospholipase A may show weak activity as a direct haemolysin when added to washed red cells in the absence of added phospholipid. This activity has been attributed to the action of phospholipase A on that portion of the phospholipid of the red cell which is in a form loosely bound to the cell surface. Loosely bound phospholipid has been demonstrated by Lovelock (1955), who showed by successive washings with 'saline' that significant amounts of phospholipid could be readily removed from the red-cell membrane. The venom of *Notechis scutatus* has a markedly low activity as a direct haemolysin (Table 1). This fact and the agreement between the recovery of both the direct haemolytic and phospholipase A activity of this venom as shown in Table 2 lead us to believe that the venom of *Notechis scutatus* has no significant direct haemolytic activity other than that due to phospholipase A.

In the first step in the separation of phospholipase A from the venom of *Pseudechis porphyriacus*, namely adsorption on barium carbonate, the removal of cholinesterase, among other enzymes, is

of interest. This step removed less than 40 % of the cholinesterase from the venom of *Notechis scutatus* (Doery, 1958), in which the concentration is 60 times that found in the venom of *P. porphyriacus*. Thus, although barium carbonate removed specifically the clotting factor, a number of other enzymes occurring in small concentrations were also removed. It would seem possible that the extent to which they are removed depends on the concentration in which they occur in the venom. The only other enzyme of those studied which was present at this stage was 5′-nucleotidase. We have no indication whether the subsequent low recovery was due to instability or adsorption on the resin.

The separation of fraction X-2, with seven times the phospholipase A activity of the crude venom, provided a high concentration of this enzyme free from the other main enzymes of the venom. However, the distribution of phospholipase A activity in fraction X-2, as shown in Fig. 2, suggests that within this fraction itself there may be two phospholipase A components. These were not separated in the ultracentrifuge. From the results presented here, it is evident that phospholipase A activity is associated with still other fractions of the venom. Although the paper-electrophoresis patterns of fractions X-1 and Y overlap with that of fraction X-2 (Figs. 3–6), the differences which have been demonstrated by chromatography, dialysis and possible substrate or activation specificities lead us to believe that the venom of *P. porphyriacus* contains a number of components with phospholipase A activity. Attention has already been drawn to the occurrence of two or more enzymes of the same type within the one source (Doery, 1958).

The possible relationship between the phospholipase A-rich fractions of the venom of *P. porphyriacus* and those from other venoms was considered. Fraction X-1 is inhomogeneous, represents only 3 % of the activity of the crude venom, and might be considered of little significance but for the fact that it is consistently separated from this venom, and has similar properties to the phospholipase A-rich fraction of the venom of *Notechis scutatus*. The latter fraction was recently re-examined under the chromatographic conditions which were used here to separate fractions X-1 and X-2. It behaved entirely like fraction X-1 with no indication of a component like X-2. This fact, taken together with both the similarity in paper-electrophoresis pattern (Figs. 3 and 4) and similar loss of activity on dialysis, supports the belief that there is a close relationship between fraction X-1 and the phospholipase A-rich fraction of the venom of *Notechis scutatus*.

The phospholipase A prepared from the venom of *Crotalus terrificus* (Neumann & Habermann, 1955; Habermann, 1957) had an isoelectric point near pH 6·5, was strongly adsorbed to the resin Amberlite IRC-50 at pH 6·86 and lost activity on dialysis. No single fraction of the venom of *P. porphyriacus* can be identified with this preparation.

Possible fundamental differences between the three fractions of the venom of *P. porphyriacus* containing phospholipase A have been suggested from the experiments concerning substrate specificities. The lower activity of fraction X-2 against egg lecithin than against whole egg yolk as substrate could be explained by the presence of an activator in egg yolk that was specific for fraction X-2. A limited examination suggested that sodium oleate may contribute to such activation.

SUMMARY

1. Both phospholipase A and direct haemolysin are found among the common venomous species of Australian snakes, phospholipase A being more generally distributed. The venoms of three species of *Pseudechis* genus, namely *porphyriacus*, *papuanus* and *australis*, are rich in both haemolysins.

2. In the venoms of *Notechis scutatus scutatus*, *Oxyuranus scutellatus*, *Acanthophis antarticus* and *Demansia textilis textilis*, the direct haemolysin was either not detected or present in small concentrations compared with that of the venom of *Pseudechis porphyriacus*.

3. A number of fractions containing phospholipase A activity were separated from the venom of *P. porphyriacus*. One of these contained sevenfold the activity of the crude venom. A family of enzymes is indicated, with possible substrate or activation specificities.

4. The relationship of these preparations to phospholipase A prepared from other venoms is discussed.

We wish to thank the Director of Ross Allen's Reptile Institute, Florida, U.S.A., and the Director of the Medical Research Institute, Johannesburg, South Africa, for gifts of venoms. We thank Miss M. Gilbo for carrying out the paper electrophoresis, Dr I. J. O'Donnell for the ultracentrifuge analysis and Miss F. Bates and Miss S. Marklein for skilled technical assistance. This work was reported in part to the 4th Annual Meeting of the Australian Biochemical Society, Perth (1959).

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The Partition of Solutes between Buffer Solutions and Solutions Containing Hyaluronic Acid

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(*Received* 24 *August* 1960)

This paper describes experiments which show that hyaluronic acid may affect markedly the partition of diffusible solutes between solutions that contain it and buffer solutions; this effect can be described as the exclusion of the solute from a part of the volume of solution occupied by the hyaluronic acid. Johnston (1955) suggested the occurrence of a large degree of exclusion to explain the effect of hyaluronic acid on the ultracentrifugal sedimentation of serum albumin; however, Blumberg & Ogston (1956) were able to find only a small effect, by observing the effect of albumin on the sedimentation of hyaluronic acid. Aldrich (1958) observed directly a small degree of exclusion of raffinose by hyaluronic acid.

In spite of Blumberg & Ogston's result, steric considerations suggested that with large solute molecules quite large degrees of exclusion might be expected; such exclusions would be of considerable interest in relation to the partition of solutes between blood and tissue spaces believed to contain hyaluronic acid (reviewed by Rogers, 1961). Moreover, the occurrence of mutual exclusion is familiar in the physical chemistry of high polymers (Flory, 1943; Scott, 1949). Accordingly, we decided to investigate the effect by the direct method of dialysing solute to equilibrium between buffer solutions and solutions containing hyaluronic acid.

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EXPERIMENTAL

Methods of dialysis

β-Dextrin. As this solute passes easily through cellophan, a method like that of Aldrich (1958) was used. Since β-dextrin could not be estimated accurately in the presence of hyaluronic acid (because the latter reacts to some extent with periodate), only the buffer fluid was analysed after equilibration. The amount of β-dextrin in the hyaluronic acid solution was obtained by subtracting from the total β-dextrin that remaining in the buffer. Since the volume of the hyaluronic acid solution might change a little during dialysis, this was estimated by mopping buffer off the sac which contained it (at the end of dialysis), weighing the sac and subtracting the weight of the wet cellophan. A reasonably accurate estimate of the concentration of β-dextrin in the hyaluronic acid solution was thus made.

Other solutes. Instead of cellophan, Millipore HA membrane, of average pore diamter 0·45 μ, was used (Millipore Filter Corp., Watertown, Mass., U.S.A.). The membrane separated two compartments each of 5 ml. capacity, in the Perspex apparatus shown in Fig. 1. All the

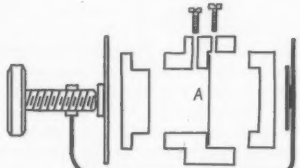

Fig. 1. Diagram of dialysis cell and clamp. *A*, Millipore membrane; *B*, filling plugs.

solutes used dialysed readily through this membrane, whereas hyaluronic acid was completely retained. When the compartments were filled, by syringe, a small air bubble was left in each, and stirring was effected by rotating the whole apparatus about its axis at 1 rev./min. The solute investigated was present initially in one or other compartment, usually in the buffer. Dialysis took up to 6 days at room temperature. At the end of equilibration, both solutions were withdrawn for analysis.

Light-scattering

Light-scattering measurements on a sample of hyaluronic acid complex were made by Dr B. N. Preston of this Department, using a Brice–Phoenix light-scattering apparatus. The hyaluronic acid was clarified by centrifuging it at a concentration of 0·14 g./100 ml. for 8 hr. at 30 000 rev./min. in the 30 head of a Spinco Model L ultracentrifuge. Measurements were made in the standard buffer over the concentration range 4–64 mg./100 ml. The results were calculated from the Zimm plot (Zimm, 1948).

Sedimentation

Sedimentation was observed in the Svedberg oil-turbine ultracentrifuge at 900 rev./sec. and 26°, by the method of Cecil & Ogston (1948) with the bar-schlieren optics of Baldwin (1953).

Materials

Cellophan tubing. This was Visking 1 cm. tubing. Before use it was thoroughly washed in water, to ensure that it contained no soluble material reacting with periodate.

Millipore membranes. It was found necessary to wash these before use only in experiments with inulin. Fresh membranes gave rise to soluble material which analysed as fructose and even exhaustive washing did not entirely remove this. However, in control experiments, it was established that the soluble material distributed itself equally between hyaluronic acid solutions and buffer. Correction could therefore be applied by assuming equipartition of any material in excess of the total inulin added.

Hyaluronic acid. The material used throughout was the hyaluronic acid–protein complex, prepared from ox synovial fluid by the filtration method of Ogston & Stanier (1950). The washing process was repeated 10–20 times. The product contained no free protein.

Bovine-serum albumin. Bovine-serum albumin (The Armour Laboratories, Hampden Park, Eastbourne, Sussex) batch no. M 12007, was used.

Carbon monoxide-haemoglobin. This was prepared from washed erythrocytes of horse blood saturated with CO. The cells were lysed by dilution of the suspending fluid with water, and the stroma was removed by centrifuging at 60 000 *g* for 1 hr.

β_A-Lactoglobulin. This was prepared from identified milk essentially as described by Ogston & Tombs (1957).

Tropomyosin. This was prepared from rabbit muscle by the method of Bailey (1948).

β-Naphthol-3:6-disulphonic acid azodiphenylazo derivatives of proteins. (R-salt proteins). The preparation of the diazonium chloride from benzidine and β-naphthol-3:6-disulphonic acid (R-salt) and the coupling with proteins were carried out as described by Kabat & Heidelberger (1937). For 1 g. of serum albumin the equivalent of 0·11 g. of benzidine was used; for 1 g. of β_A-lactoglobulin the equivalent of 0·09 g. of benzidine was used. The resulting solutions of highly coloured proteins were exhaustively dialysed against buffer and any insoluble material was removed by centrifuging. Stock solutions contained 0·1 g. of coupled protein/100 ml.

Dinitrophenyl derivatives of protein. Preliminary experiments showed that the reaction of small quantities of 1-fluoro-2:4-dinitrobenzene was complete in 48 hr. at 2° in 0·067 M-Na_2HPO_4 containing 5% ethanol (v/v), about half the total fluorodinitrobenzene reacting with the protein. To 1 g. of protein in 40 ml. of 0·067 M-Na_2HPO_4 was added 10·6 mg. of fluorodinitrobenzene in 2 ml. of ethanol; this introduces 1–2 equiv. of dinitrophenyl/mole of serum albumin. After 100 hr. at 2°, the mixture was acidified with 3–4 m-equiv. of HCl (pH approx. 4) and extracted ten times with 45 ml. of light petroleum (b.p. 60–80°). The last traces of light petroleum were removed by suction, with the solution at 35°. The solutions were exhaustively dialysed against buffer and any insoluble material was removed by centrifuging. Dinitrophenyl derivatives of bovine-serum albumin, β_A-lactoglobulin and tropomyosin were made.

Polyglucose. Synthetic polyglucose was a present from Dr P. T. Mora (Mora, 1958). The sample had been precipitated by ethanol between 62·5 and 72·5% (v/v). It was stated to have a number-average molecular weight of 50 000 by osmotic pressure and of 21 000 by measurement of reducing end-groups. Light-scattering measurements made by Dr P. Johnson (personal communication) gave a weight-average molecular weight of 33 000 with an error of ±3000. The sample was labelled with ^{14}C by use of $K^{14}CN$ (The Radiochemical Centre, Amersham, Bucks.) by the procedure of Isbell (1953).

Inulin. Four preparations of inulin were used. Sample (A) was Kerfoot biochemical reagent inulin (Thomas Kerfoot Ltd., Vale of Bardsley, Lancs), twice recrystallized by dissolving it in dilute NH_3 soln. [0·1 ml. of NH_3 soln. (sp.gr. 0·88)/100 ml. of water], filtering off insoluble material and cooling to 1°. Sample (B) was reagent quality (British Drug Houses Ltd.), twice recrystallized, first after solution in hot 0·1 M-NaOH, then as above. Sample (C) was a present from Dr D. J. Bell, prepared by him and recrystallized as sample (A). Sample (D) was inulin from British Drug Houses Ltd. heated in glycerol for 20 min. and cooled; ethanol was then added to 30% (v/v) and the precipitate was discarded; then ethanol was added to 50% (v/v) and the precipitate was collected. Inulin was dried in air at 105° and finally exhaustively *in vacuo* over P_2O_5.

Synthetic amyloses of degrees of polymerization (D.P.) of 19 and 38. These were a present from Dr W. J. Whelan (Whelan & Bailey, 1954).

Schardinger β-dextrin. This was a present from Dr P. W. Kent.

Buffers and measurement of pH. All measurements except those with the β-dextrin were made in 0·2 M-NaCl, 7·7 mM-Na_2HPO_4, 2·3 mM-KH_2PO_4, pH 7.3. Solutions of hyaluronic acid complex and of proteins were exhaustively dialysed against large volumes of this buffer. Measurements of pH were made with a Cambridge pH meter, standardized with 0·05 M-potassium hydrogen phthalate (pH 4·0) and 0·05 M-sodium borate (pH 9·24).

Methods of analysis

Hyaluronic acid complex. The concentration was determined by drying to constant weight at 105° after thorough

dialysis against distilled water. Closed Visking sacs were used for dialysis; any change of volume during dialysis was checked by direct weighing of the sacs after mopping them free from excess of liquid.

Coloured proteins. It was necessary to use these in the partition experiments to distinguish the partitioned protein from that present in the hyaluronic acid complex. The latter showed no absorption at the wavelengths at which the coloured proteins were estimated in a Hilger Uvispek spectrophotometer: carbon monoxide–haemoglobin at 570 mμ, R-salt proteins at 527 mμ, dinitrophenyl proteins at 360 mμ.

Polyglucose. This was estimated by radioactive counting. In order to allow for self-absorption due to hyaluronic acid, solutions from the buffer compartment were diluted with a solution of hyaluronic acid complex before estimation. Solution from the hyaluronic acid-complex compartment was similarly diluted with buffer, so that both final solutions contained the same concentration of salts and of hyaluronic acid complex. Samples (0·05 ml.) of solution were transferred to circles of lens tissue (diameter 16 mm.) on 19 mm. coverslips and counted with a mica end-window tube. The counting rate was about 3000 counts/min. A minimum of 10 000 counts were obtained.

Inulin. This was estimated by the resorcinol method essentially as described by Kulka (1956). Inulin (5–50 μg. in 2 ml.) was heated for 40 min. at 80° with 3 ml. of resorcinol in ethanol (0·05%, w/v) and 3 ml. of HCl (sp.gr. 1·18). Extinctions were measured at 480 mμ after cooling. Where hyaluronic acid complex was present deproteinization was necessary. Metaphosphoric acid (7%, w/v) was satisfactory for this; 1 ml. was added to 2·5 ml. of solution of inulin and hyaluronic acid, and the solution was centrifuged for 15 min. at 10 000 *g* before estimation. Control experiments showed recoveries of 99±1% of inulin. There was still a small error due to the hyaluronic acid, amounting to not more than 2% of the extinction due to inulin, and correction for this was made. It has been mentioned that the Millipore membranes gave rise to small amounts of material reacting as fructose, even after exhaustive washing. Control dialysis experiments, without inulin, showed that this material is equally partitioned between buffer and hyaluronic acid. Correction was therefore made by assuming equal partition of excess of apparent inulin over that known to be present (after allowing for the contribution of the hyaluronic acid) by subtracting half the apparent excess of inulin from the amount estimated in each compartment.

Amyloses. These were estimated by the method of Whelan & Bailey (1954) by use of 0·2% (w/v) I_2 in 2% (w/v) KI in water. D.P. 19 amylose was measured at 510 mμ and D.P. 38 amylose at 560 mμ, after dilution to 40–80 μg./ml. of final solution. Neither hyaluronic acid nor any material from the Millipore filters interfered.

Schardinger β-dextrin. The estimation was based upon the reaction with metaperiodate (Aspinall & Ferrier, 1957). The following conditions were most satisfactory: Dextrin (2 ml., 0·1 g./ml.) was added to freshly made $NaIO_4$ (5 ml., 0·015 M) with a crystal of KCl and incubated under N_2 in a Thunberg tube in the dark at 35° for 10 hr. Under these conditions the reaction was complete and there was little loss of periodate in the absence of dextrin. The presence of phosphate in the standard dialysis buffer led to erratic results, but reproducible results were obtained in the presence of NaCl alone: accordingly, these dialyses and estimations were performed in 0·2 M-NaCl. The extinctions of the original and remaining periodate, and of equimolar iodate, were read at 223 mμ. Hyaluronic acid complex was found to react with periodate over long periods to too great an extent to make correction for its reaction reliable. Small quantities of material reacting with periodate were found in the diffusate on prolonged dialysis of hyaluronic acid complex against buffer (without dextrin). Reliable correction could be made for this by use of a relatively high concentration of dextrin.

RESULTS

The first partitioned solute to be investigated was inulin, chosen because of its use in the measurement of extracellular volumes *in vivo* and *in vitro*. Preliminary experiments showed a surprisingly unequal partition, and this effect was fairly thoroughly investigated. Once it had been established that the effect is real and reproducible, other solutes required less extensive investigation. The results are described more or less in the order in which they were obtained.

Partition of inulin

Time course and direction of dialysis. Fig. 2 shows that the partition of inulin approached a stable end-point, and that this was the same whether the inulin was initially added to the buffer or to the hyaluronic acid solution.

Effect of different samples of inulin. The partitions of different samples under identical conditions are shown in Table 1. The sample found otherwise to contain material of relatively low molecular weight showed less unequal partition (see below): the 'good' samples show satisfactory agreement.

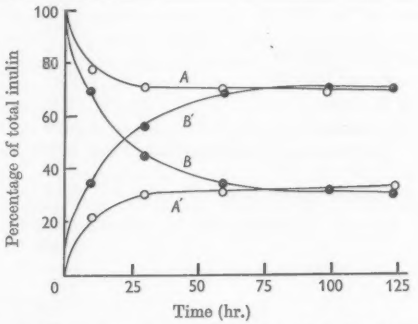

Fig. 2. Time courses of equilibration of inulin between buffer solution and a 0·18% solution of hyaluronic acid complex. Curves *A* and *A′* were with the inulin initially in the buffer: *A*, loss of inulin from the buffer; *A′*, gain of inulin by the hyaluronic acid solution. Curves *B* and *B′* were with the inulin initially in the hyaluronic acid solution: *B*, loss of inulin from the hyaluronic acid solution; *B′*, gain of inulin by the buffer.

Effect of varying the ionic strength and pH. Closely concordant results were obtained with 0·18% hyaluronic acid complex and inulin sample (A) (50 μg./ml.) in the standard buffer but containing NaCl up to 2M, and in 0·2M-NaCl with pH varied from 6 to 9 by phosphate and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffers.

Effect of varying the concentration of inulin. With hyaluronic acid complex (0·18%) and standard buffer, the same result was obtained with inulin sample (A) concentrations from 25 to 250 μg./ml.

Effect of varying the concentration of hyaluronic acid. The partition of sample (A) varied with the concentration of hyaluronic acid complex as shown in Table 2.

Table 1. *Partition of different samples of inulin between buffer and solution of hyaluronic acid complex*

Concn. of hyaluronic acid complex was 0·18%; concn. of inulin initially was 50 μg./ml. in standard buffer; dialysis was for 100 hr.

Sample of inulin	Percentage of total inulin in	
	Buffer	Hyaluronic acid
From T. Kerfoot Ltd., unpurified	60	40
From T. Kerfoot Ltd., recrystallized (A)	69	31
From British Drug Houses Ltd., recrystallized (B)	68	32
From D. J. Bell, recrystallized (C)	70	30
Ethanol-fractionated (D)	68	32

Properties of samples of inulin

Comparison of crude inulin (Kerfoot) with recrystallized samples showed that it appeared to contain significant amounts of material of lower molecular weight. (i) The crude material passed through Visking tubing much more rapidly than the recrystallized material. (ii) Descending chromatography on Whatman no. 1 paper in butan-1-ol-pyridine–water (6:4:3, by vol.) showed the crude material to be markedly heterogeneous. (iii) The crude material showed a higher apparent solubility in water at 25°. (iv) The crude material showed a considerable fraction not precipitated by less than 50% (v/v) ethanol. It is possible that the

Table 2. *Partition of solutes between buffer and solutions of hyaluronic acid complex*

All dialyses were in standard buffer except for β-dextrin (see text). K = (concn. in buffer)/(concn. in hyaluronic acid).

Solute	Concn. of hyaluronic acid complex (%)	Dialysis time (hr.)	Percentage of total solute in		K
			Buffer	Hyaluronic acid	
Inulin (A)	0·18	100	69	31	2·22
	0·09	100	61	39	1·56
	0·045	100	56	44	1·27
	0·023	100	53	47	1·13
Polyglucose	0·18	163	61	39	1·56
	0·09	163	55	45	1·22
	0·045	163	52	48	1·08
Amylose D.P. 19	0·18	160	59	41	1·44
Amylose D.P. 38	0·18	160	65	35	1·85
β-Dextrin	0·32	192	52	48*	1·08
Raffinose†	0·28	192	50·5	49·5	1·02
Serum albumin (R-salt deriv.)	0·18	140	72	33	2·18
	0·09	140	63	36	1·75
Dinitrophenyl serum albumin	0·18	160	72	28	2·57
	0·09	160	64	36	1·77
	0·045	160	58	42	1·38
	0·023	160	54	46	1·17
β-Lactoglobulin (R-salt deriv.)	0·18	140	66	34	1·94
	0·09	140	58	42	1·38
	0·045	140	53	47	1·13
Dinitrophenyl β-lactoglobulin	0·18	160	67	33	2·03
Dinitrophenyl tropomyosin	0·18	120	73	27	2·70
	0·09	120	62	38	1·63
	0·045	120	57	43	1·33
Carbon monoxide–haemoglobin	0·18	160	67	33	2·03
	0·09	160	59	41	1·44
	0·045	160	52	48	1·08

* Not determined directly, see text.

† From Aldrich (1958).

purified samples are not identical, since cursory examination showed that their apparent solubilities and rates of passage through Visking cellophan differ; the latter may, however, have been due to variations of Visking cellophan, even when pieces were taken from the same roll (Dr W. J. Whelan, personal communication).

Partition of serum albumins

With the R-salt protein, it was established that dialysis for 140 hr. was sufficient to establish equilibrium. Subsequent experiments with the dinitrophenyl albumin showed that the partition was not affected by the direction of dialysis, by increase of salt concentration up to 2 M-NaCl, by variation of pH between 6 and 9 or by the concentration of protein between 0·1 and 2 g./100 ml. The effect of changing the concentration of hyaluronic acid was similar to that on inulin, and is shown in Table 2.

Partition of other solutes

The results are summarized in Table 2. In each case it was established that the time of dialysis was sufficient to attain equilibrium. In all but one of these experiments the solute was initially in the buffer. One experiment with polyglucose initially in the hyaluronic acid compartment gave a result close to that in which it was initially in the buffer.

Sedimentation of hyaluronic acid in the presence of β_A-lactoglobulin

These experiments were done because the partition experiments appear to contradict the conclusion of Blumberg & Ogston (1956) that there is only a small exclusion of protein by hyaluronic acid. β_A-Lactoglobulin was chosen as the protein because its sedimentation coefficient (2·8s) is much less than that of hyaluronic acid at low concentration of the latter. The sedimentation rate of hyaluronic acid (at a concentration of 0·016 %) was measured in the presence of various concentrations of protein. The results are shown in Table 3.

Table 3. *Sedimentation of hyaluronic acid complex in the presence of β_A-lactoglobulin*

Concn. of hyaluronic acid complex was 0·16 %. Speed was 900 rev./sec.; temp. 26°; sedimentation coefficients are corrected to 20° in the standard buffer.

Concn. of β_A-lactoglobulin (g./100 ml.)	Sedimentation coefficient of hyaluronic acid complex (s)
0	5·86
0·065	5·84
0·13	5·95
0·33	5·96

Light-scattering by the hyaluronic acid complex

A consistent Zimm plot was obtained, indicating a molecular weight of 13×10^6, a radius of rotation of 3750 Å and a second virial coefficient of $3{\cdot}7 \times 10^5$ c.g.s. units. These values are in fair agreement with those obtained on hyaluronic acid complex by dynamic methods (Blumberg & Ogston, 1958) and on suitable preparations of protein-free hyaluronic acid from ox synovial fluid (Balázs, 1958).

DISCUSSION

Exclusion of solutes by hyaluronic acid

It seems to be established by these experiments that solutes are unequally partitioned between buffer solutions and solutions containing hyaluronic acid complex, by what amounts to a partial exclusion of solute by the hyaluronic acid from the solution occupied by the latter. One may inquire first what is the general nature of this exclusion. The facts that the partition is quantitatively independent of ionic strength and pH of the solution, that it is not obviously affected (as between different proteins) by the isoelectric point, that there is a general relationship between partition coefficients (K) and the molecular sizes irrespective of the chemical nature of the solute, and that it is strongly affected by the concentration of hyaluronic acid, all point to the essentially steric nature of the exclusion. Hyaluronic acid is a chain polymer of very large molecular weight and of random chain configuration (Blumberg & Ogston, 1958; Balázs, 1958). The radius of its molecular domain is of the order of 3500–4000 Å, which means that each molecule occupies a volume of 10–15 l./g. At concentrations down to 0·01 % there will therefore be an extensive interpenetration of neighbouring chains, and at concentrations of the order of 0·1 % and upward a solution of it will consist of an almost uniform network of polysaccharide chain. It is clear, qualitatively, that a compact solute

Table 4. *Values calculated for R_B of hyaluronic acid from values of R_S, β and equation 2: see text*

Solute	β	R_S (Å)	R_B (Å)
Raffinose	3	4·9	4·1
β-Dextrin	11	6·5	2·8
Polyglucose	98	20*	3·3
β_A-Lactoglobulin	160	22	3·1
Carbon monoxide–haemoglobin	170	27	3·6
Serum albumin	250	27	3·1
Amylose, D.P. 19	88	9·0 (19)	1·6
Amylose, D.P. 38	150	11·4 (23)	1·6
Inulin	204	10·6 (26)	1·4
Tropomyosin	270	30 (29)	3·4

* Average mol.wt. taken as 33 000.

particle will be able to fit into only limited sites in this network, the fewer sites the larger the particle.

Ogston (1958) has given a geometrical treatment of the distribution of 'holes' in a random suspension of straight fibres, calculating the probability $P_{D>r}$ that an object of radius r can be placed at any point within the network. Since the partition coefficient $K = 1/P_{D>r}$ this theory could be applied to the present case by making the assumptions (i) that the hyaluronic acid chains consist of randomly-kinked straight segments, (ii) that the number of 'ends' is equal to the number of kinks. Knowing approximately the effective radius of a solute particle, the average length of straight segments can then be calculated from the partition coefficient and equation (13) from Ogston (1958). Unfortunately, this leads consistently to segment lengths of the order of 1 Å, which is absurd.

This result does, however, suggest another similar approach to the problem, which is to treat the hyaluronic acid chain as a string of beads which can be regarded (approximately) as randomly distributed in solution: this is equivalent to a modification of Ogston's treatment of fibres, by treating them as all 'ends' and leads directly to

$$1/P_{D>r} = K = \exp[4\pi\nu r^3/3], \qquad (1)$$

when ν is now the number of beads/ml. and r is the effective radius of the solute particle. This radius is the sum of the particle radius R_S and that of the bead R_B. If $\bar{v}$ is the partial specific volume of the beads, equation (1) becomes

$$\log K = \frac{\bar{v}}{2{\cdot}303}\left\{\frac{R_B+R_S}{R_B}\right\}^3 w = \beta w, \qquad (2)$$

where w is the weight of hyaluronic acid/ml. and β is a constant for a given solute. Fig. 3 shows that this relationship is approximately obeyed.

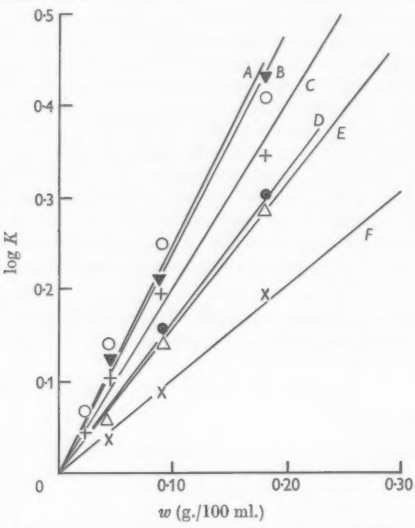

Fig. 3. Plots of log K against w for various solutes (see text). A (▼), Tropomyosin; B (○), serum albumin; C (+), inulin; D (●), carbon monoxide–haemoglobin; E (△), β_A-lactoglobulin; F (×), polyglucose.

Table 4 shows values for R_B calculated from the values of β, $\bar{v}$ (taken as 0·69, Ogston & Stanier, 1950) and of R_S. The last are calculated on the assumption that the solute molecules are spherical, compact and unsolvated (the partial specific volumes are taken as 0·75 for proteins and 0·60 for carbohydrates). In Table 4 the resulting values of R_B are collected into two sets, the first those for which the last assumption is likely to be approximately correct, and the second for solutes believed not to be of spherical or compact configuration. With the exception of tropomyosin in the second half, the values of R_B divide well into high and low. The values in the first half give an average for R_B of 3·34 Å, corresponding to a bead weight of 158. The approximate correspondence of this with that of a hexose unit must be fortuitous, but the value is at least a reasonable one for the 'bead model', and gives some sort of picture of steric exclusion.

The value for R_B of 3·34 Å for the compact particles may now be used to calculate R_S for the others: this gives the values in parentheses in the second half of Table 4. The three linear polysaccharides are seen to have effective radii much larger than those of the corresponding compact spheres. This is not unexpected, since such molecules are likely to be elongated, with a considerable degree of stiffness, and the size of the 'hole' required will be more closely related to the volume of rotation than to the molecular volume. By contrast polyglucose has a highly branched and relatively compact structure. The behaviour of tropomyosin seems to be anomalous since all the evidence (Bailey, 1954) suggests that it is a stiff molecule of large axial ratio.

Equations (1) and (2) show that the 'excluded volume' is not a constant quantity per weight of polymer (for a given solute) but varies with the concentration of polymer:

$$\text{Excluded vol./wt. of polymer} = \frac{1}{w}\left\{1-\frac{1}{K}\right\} = \frac{1}{w}\{1-10^{-\beta w}\}. \qquad (3)$$

Effect of protein on the sedimentation of hyaluronic acid

Blumberg & Ogston (1956) pointed out that exclusion of solute by hyaluronic acid would amount to selective solvation of the hyaluronic acid by

solvent, so that a large exclusion should markedly affect the sedimentation of the hyaluronic acid. From their observations on an ascending boundary of hyaluronic acid, at a concentration of 0·28 %, in the presence of serum albumin, they concluded that the exclusion could not under those conditions be more than 12 ml./g. of hyaluronic acid. By contrast, the present partition results, with use of equation (3), would predict an exclusion of nearly 300 ml./g. under their conditions. Our observations on the downward-moving boundary of hyaluronic acid in the presence of β_A-lactoglobulin qualitatively confirm those of Blumberg & Ogston in showing that the protein has far less effect than would be expected from the partition experiments.

The only possible explanation of this discrepancy seems to be that an equilibrium partition of protein in relation to hyaluronic acid is not nearly attained under the conditions of sedimentation, but that protein moves with the hyaluronic acid in excess of expectation.

Distribution of substances in tissue spaces and the estimation of extracellular volumes

A review of these questions would be out of place here. It is enough to point out that our experiments make it highly probable that macromolecular substances, in particular proteins and polysaccharides of the type of inulin, will be very unequally distributed between blood or incubation fluids and tissue spaces wherever the latter contain significant concentrations of hyaluronic acid or similar material. The effect of this will be to cause extracellular water to be underestimated. The normal distribution of protein *in vivo*, between blood or lymph and tissue spaces, will be similarly affected. It cannot be assumed, for example, that the concentration of serum protein in lymph is the same as that in extracellular water from which the lymph was derived; in general, it will tend to be greater. The situation here is likely to be complicated also by resistance offered by a polysaccharide network to the passage of solutes from blood to lymph through the tissue spaces.

SUMMARY

1. The partition of a number of diffusible solutes, including inulin and proteins, between buffer solutions and solutions of hyaluronic acid has been examined.

2. All substances examined are partitioned in favour of buffer, increasingly with increase of effective molecular volume.

3. The importance of these findings to the distribution of natural and artificial macromolecular substances between fluids and tissue spaces, *in vivo* and *in vitro*, and to the use of such substances for measuring extracellular volumes, is pointed out.

4. A simple steric theory is put forward to explain this effect.

We are grateful to the Medical Research Council for a Training Studentship held by one of us (C.F.P.), to Dr D. J. Bell, Dr P. W. Kent, Dr P. T. Mora and Dr W. J. Whelan for gifts of materials, and to Dr W. Bartley for advice.

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Condensed Tannins

9. DISTRIBUTION OF FLAVONOID COMPOUNDS IN THE HEARTWOODS AND BARKS OF SOME INTERRELATED WATTLES

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(*Received* 22 *July* 1960)

The flavan-3-ols (−)-robinetinidol, (+)-catechin and (+)-gallocatechin occur in the bark (Roux, 1953; Roux & Maihs, 1958, 1960*a*), whereas (+)-7:3′:4′-trihydroxyflavan-3:4-diol (Keppler, 1957), (+)-fustin, (−)-fisetinidol, fisetin and polymeric leuco-fisetinidin tannins (Roux & Paulus, 1961*a*, *b*) are prominent heartwood components of the commercially afforested black-wattle tree [*Acacia mollissima*, recently renamed *A. mearnsii* (cf. Brenan & Melville, 1960)]. Of the four *Acacia* spp. introduced into South Africa from Australia for their tannin-rich bark, *A. mearnsii* is closely related to *A. decurrens* (green wattle) and to *A. dealbata* (silver wattle), all having feathery compound leaves of similar appearance. *A. pycnantha* (golden wattle) with leaves in the form of lanceolate phyllodes is known to be more distantly related to this group. In the present study the distribution of flavonoid compounds in the barks and heartwoods of these species is compared.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points are performed on molecular mixtures (Roux & Maihs, 1960*a*). Analyses of C and H were by Weiler and Strauss, Oxford. Infrared comparisons were by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. A 2 dm. tube was used for optical rotations. Two-dimensional chromatograms were run in water-saturated butan-2-ol for direction 1 and in 2% acetic acid for direction 2.

Isolation, identification and estimation of heartwood components

Acacia decurrens and *Acacia dealbata*. On two-dimensional chromatograms the methanol-soluble heartwood components of green and silver wattles show the same pattern of components as those present in black-wattle (*A. mearnsii*) heartwood extractives. Those heartwood components which have been isolated from black wattle, namely (+)-7:3′:4′-trihydroxyflavan-3:4-diol, (+)-fustin, (−)-fisetinidol and fisetin (Roux & Paulus, 1961*a*, *b*), were used to identify the corresponding components in *A. decurrens* and *A. dealbata* by means of two-dimensional paper chromatography. The addition of the above-mentioned compounds caused increases in the concentration of the corresponding substances in each of the two extracts. Identity of the compounds was confirmed by colour reactions with toluene-*p*-sulphonic acid, ferric alum and bis-diazotized-benzidine spray reagents. The concurrence of spots and colour reactions were considered as sufficient proof of identity. Polymeric leuco-fisetinidin tannins occurred in each heartwood.

Acacia pycnantha. Golden wattle, although related to the above group, shows some divergent botanical characteristics. The pattern of heartwood components of *A. pycnantha* was almost identical with those of black, green and silver wattles but showed slight differences. Golden-wattle-heartwood components were accordingly isolated to ensure their identity.

Drillings (4·3 kg.) from the dry heartwood of golden-wattle trees from the Union Government Plantations at Eerste River, near Cape Town, were extracted with methanol in three successive extractions of 48 hr. duration each, to yield 213 g. of solids. The solids were dissolved in hot ethanol (500 ml.) and chloroform (400 ml.) was added to precipitate tannins. The solubles (130 g.) were fractionated by solution in 400 ml. of ethyl acetate–ethanol (2:1) followed by the addition of chloroform (300 ml.) with shaking. The solids remaining after evaporation of the filtrate (83 g.) were resolved in a Craig machine by the method described by Roux & Paulus (1961*b*). After 160 transfers fisetin (140–160), fustin (105–135), fisetinidol (105–125) and (+)-7:3′:4′-trihydroxyflavan-3:4-diol (44–69) were located in the tubes indicated by means of two-dimensional paper chromatography. The contents of the appropriate tubes were grouped together and the compounds isolated by separation on cellulose sheets as described by Roux & Paulus (1961*a*, *b*). Fisetin (20 mg.), fustin (2·3 g.), fisetinidol (150 mg.) and 7:3′:4′-trihydroxyflavan-3:4-diol (900 mg.) were obtained in a crystalline condition.

(*a*) Fisetin. The solids present in tubes 141–160 were boiled with N-H_2SO_4 and the insoluble solids extracted with ether in a Soxhlet apparatus. The ether extractives were recrystallized from ethanol–water; m.p. and mixed m.p. with fisetin prepared from (±)-fustin of *Rhus glabra* (Roux & Paulus, 1961*a*) 320°. $\lambda_{max.}$ 252, 320 and 360 mμ in ethanol.

(*b*) (+)-Fustin. The fustin was recrystallized from water, m.p. and mixed m.p. with (+)-fustin from *A. mearnsii* (Roux & Paulus, 1961*a*) 214–216°. $[\alpha]_D^{23}$ +21·5±2·2° in acetone–water (1:1) (*c*, 0·8). The tetra-acetyl derivative was prepared as before, m.p. and mixed m.p. with tetra-acetyl-(+)-fustin 117–119°. $[\alpha]_D^{23}$ +22·9±0·1° in tetra-

* Part 8: Roux & Paulus (1961*b*).

chloroethane (*c*, 1·2). The low m.p. of the tetra-acetyl derivative compared with that of the racemate (147–151°), and the high optical rotations, indicate that optically pure (+)-fustin is present also in *A. pycnantha* heartwood.

(*c*) (−)-Fisetinidol. The fisetinidol was recrystallized from water (charcoal), m.p. and mixed m.p. with (−)-fisetinidol from *A. mearnsii* (Roux & Paulus, 1961*b*) 212–214°. $[\alpha]_D^{22} -4{\cdot}9 \pm 0{\cdot}3°$ in acetone–water (1:1) (*c*, 0·8). In 2% acetic acid on Whatman no. 1 paper the R_F (0·48) of the fisetinidol differed from that (0·43) of synthetic (+)-fisetinidol (Weinges, 1958), but was identical with the R_F (0·48) of synthetic (−)-fisetinidol (Roux & Paulus, 1961*b*). Paper chromatography showed that only the optically pure (−)-form is present.

(*d*) (+)-7:3′:4′-Trihydroxyflavan-3:4-diol. The flavan-3:4-diol was also isolated by running 24 g. of the soluble fraction (after chloroform precipitation) on 60 sheets of Whatman no. 3 paper in 2% acetic acid and cutting the band located with the toluene-*p*-sulphonic acid spray (Roux & Evelyn, 1958*b*). Yield, 0·7 g. The flavan-3:4-diol was recrystallized from water, m.p. and mixed m.p. with (+)-mollisacacidin (Keppler, 1957; Clark-Lewis & Roux, 1959) 129°. $[\alpha]_D^{21} +30{\cdot}7 \pm 0{\cdot}8°$ in acetone–water (1:1) (*c*, 1·0). The R_F (0·54) in 2% acetic acid on Whatman no. 1 paper was identical with that of (+)-7:3′:4′-trihydroxyflavan-3:4-diol (0·54) from *A. mearnsii* (Roux & Maihs, 1960*b*), but differed from the R_F (0·48) of the (−)-enantiomer from *S. quebracho-colorado*. Infrared-absorption curves of the flavan-3:4-diols from *A. pycnantha* and *A. mearnsii* were superimposable over the range 2·5–15 μ.

(*e*) Gallic acid. A compound present in tubes 64–79 of the Craig machine was isolated by separation on cellulose sheets (30 mg.). It gave a blue colour with ferric alum spray, and a purple colour with the borate buffer (pH 7·7)–methyl red spray (Kalbe, 1954), indicating that the substance contained a pyrogallol grouping and was acidic. The compound was shown to be gallic acid by m.p. and mixed m.p. with authentic gallic acid 250°.

Estimation of flavonoid compounds in wattle heartwoods

Cross-sections of each of the heartwoods of black, green, silver and golden wattles were drilled out and the drillings were extracted with methanol (five extractions of 24 hr. duration each at room temperature). The tree samples were of different ages [black (10 years), green (9 years), silver (10 years) and golden (about 30 years)] and the sampling height above the ground is unknown. The concentration of components in the methanol extract was estimated by the paper-chromatographic–densitometric method, ammoniacal silver nitrate being used as spray reagent (Roux & Maihs, 1960*a*, *b*). Estimated percentages of different components in the four heartwoods are shown in Table 1.

Isolation, identification and estimation of bark components

The barks of the different *Acacia* species show no significant change in the composition of catechin components with increasing age, although the proportion of carbohydrate 'non-tannins' is known to decrease progressively. The average molecular weight of the tannins in the youngest strippable bark of *A. mearnsii* does not, for example, differ significantly from those in mature barks of the same tree (Evelyn, 1956). For this work 'mature' barks from the bases of 7–10-year-old trees were selected, with the exception of *A. pycnantha*, where the specimens were over 30 years old.

Acacia decurrens. Green-wattle-bark extract is apparently identical with black wattle in the composition of catechin components, differing only in the proportions of catechins present (Table 2). (−)-Robinetinidol, (+)-catechin and (+)-gallocatechin were shown to be present in green-wattle bark extractives with the chromatographic method described for heartwood components, and reference substances were obtained from black-wattle bark (Roux & Maihs, 1960*a*).

Acacia dealbata. Two-dimensional chromatograms of silver-wattle-bark extractives sprayed with bis-diazotized benzidine reagent showed the presence of (−)-robinetinidol (yellow), (+)-catechin (ochre), (+)-gallocatechin (ochre) and of a fourth substance also giving an ochre coloration. From its position on the chromatograms the latter was suspected to be (−)-*epi*catechin. Out of ten barks, from different trees examined, three showed the suspected (−)-*epi*catechin in relatively high concentration. The concentration of the other catechins was relatively constant (Table 3). The catechins were separated and identified as follows.

A portion (470 g.) of the ethyl acetate extractives (560 g.) of dry powdered silver-wattle bark [low in (−)-*epi*catechin content] was soluble on re-solution in commercial ethyl acetate. Tannins present in the soluble

Table 1. *Estimation of flavonoid compounds in wattle heartwoods*

	Concentration in methanol extractives* (%)				
Sample	(+)-7:3′:4′-Trihydroxyflavan-3:4-diol	(+)-Fustin	(−)-Fisetinidol†	Fisetin	Gallic acid
Black wattle (*A. mearnsii*)	2·0	5·1	0·6	2·0	Absent
Green wattle‡ (*A. decurrens*)	1·6	2·1	Trace	0·8	Absent
Silver wattle‡ (*A. dealbata*)	0·9	2·1	0·4	0·8	Absent
Golden wattle (*A. pycnantha*)	2·3	7·2	0·6	4·0	0·2

* Obtained from air-dried samples 2–3 years old. Compare concentrations in freshly cut *A. mearnsii* from the Grahamstown district (Roux & Paulus, 1960*a*, *b*).

† Percentages obtained by visual estimation; others were obtained by densitometric measurement.

‡ These samples contained abnormally high proportions of carbohydrate material, hence the reduced percentages of flavonoid compounds.

fraction were precipitated in batches with samples (10 g.) dissolved in 100 ml. of ethyl acetate–methanol (20:1) and adding 100 ml. of chloroform. The low-molecular-weight fraction remaining in solution after removal of the precipitated tannins was 10% of the starting material.

The enriched catechin fraction (56 g.) was streaked on to 140 sheets of Whatman no. 3 chromatographic paper (0·4 g./sheet) and the sheets were developed by upward migration in 2% acetic acid as described by Roux & Evelyn (1958*a*, *b*). Bands corresponding to (−)-robinetinidol (R_F 0·54), (+)-catechin (R_F 0·48) and (−)-*epi*catechin (R_F 0·44) were located by the bis-diazotized benzidine reagent. The bands were cut and eluted with 70% ethanol (Nordström & Swain, 1953), yielding 0·4, 5·0 and 2·2 g. of solids respectively in the eluents. The solids from each band were streaked on prewashed (with water) sheets of Whatman no. 3 paper (200 mg./sheet dissolved in 5 ml. of methanol), and the chromatograms were developed downwards in butan-1-ol–acetic acid–water (6:1:2). Bands were located, cut and eluted as before. From the eluents (+)-catechin (1·0 g.) crystallized with ease, but (−)-robinetinidol (40 mg.) and (−)-*epi*catechin (200 mg.) were run again in the partitioning mixture before crystallizing. (+)-Gallocatechin (18 mg.) was obtained from the same band (R_F 0·48 in 2% acetic acid) in which (+)-catechin was present in the initial fractionation, and crystallized after seeding with (+)-gallocatechin crystals from golden wattle.

(*a*) (−)-Robinetinidol. Robinetinidol from silver wattle was recrystallized from water, m.p. and mixed m.p. with (−)-robinetinidol (m.p. 205°) from black wattle 202°. $\lambda_{max.}$ in ethanol 202 and 282 mμ (ϵ at 282 mμ 3636) (Found: C, 56·7; H, 5·5; loss at 110°, 8·4. Calc. for $C_{15}H_{14}O_6,1{\cdot}5H_2O$: C, 56·8; H, 5·4; H_2O, 8·5%). The solution in acetone–water (1:1) was not sufficiently clear to permit measurements of optical rotation, but the robinetinidol migrated to the same position as (−)-robinetinidol on two-way chromatograms with a combination of solvent systems which separate all the stereoisomers of catechins (Roberts & Wood, 1953).

(*b*) (+)-Catechin. The substance was recrystallized from water, m.p. and mixed m.p. with (+)-catechin from *Uncaria gambir* 175–176°. $\lambda_{max.}$ in ethanol 207·5 and 281 mμ (ϵ at 281 mμ 3830) (Found: C, 59·2; H, 5·6; loss at 110°, 3·4. Calc. for $C_{15}H_{14}O_6,H_2O$: C, 58·4; H, 5·2; H_2O, 5·1%). $[\alpha]_D^{21}$ +17·0±0·1° in acetone–water (1:1) (*c*, 2·3). The acetyl derivative, m.p. and mixed m.p. with authentic penta-acetyl-(+)-catechin, 130°. $[\alpha]_D^{21}$ +29·4±0·4° in acetone (*c*, 0·9).

(*c*) (−)-*epi*Catechin. The substance was recrystallized from water, m.p. and mixed m.p. with (−)-*epi*catechin from cocoa beans (Forsyth, 1955) 235° (decomp.). $\lambda_{max.}$ in

Table 2. *Estimation of catechins in the bark extracts of different specimens of black and green wattles and of black × green wattle hybrids*

Concentration (%) in methanol extractives is given as a mean with the range in parentheses and number of samples as a superior figure.

Sample	(−)-Robinetinidol	(+)-Catechin	(+)-Gallocatechin*
Black wattle (*A. mearnsii*) on shale	0·23 (0·2–0·3)[10]	0·57 (0·5–0·65)[10]	0·32 (0·25–0·4)[10]
Black wattle (*A. mearnsii*) on dolerite	0·18 (0·1–0·25)[5]	0·51 (0·45–0·6)[5]	0·42 (0·35–0·5)[5]
Black × green wattle hybrids†	0·17 (0·1–0·25)[5]	1·15 (0·85–1·3)[5]	0·48 (0·25–0·6)[5]
Green wattle (*A. decurrens*) on shale	0·12 (0·1–0·15)[5]	1·22 (1·1–1·5)[5]	0·44 (0·4–0·5)[5]

* Estimation by visual comparison.
† Artificial hybrids produced by the Wattle Research Institute, Pietermaritzburg, Natal, during plant-breeding and genetic studies.

Table 3. *Estimation of catechins in the bark extracts of black, green, silver and golden wattles*

−, Absent.

Species	Concentration in methanol extractives (%)						
	(−)-Robinetinidol	(+)-Catechin	(−)-*epi*-Catechin	(−)-*epi*-Catechin gallate	(+)-Gallocatechin	(−)-*epi*-Gallocatechin	(−)-*epi*-Gallocatechin gallate
Black wattle* (*A. mearnsii*) (Natal)	0·3	0·2	–	–	0·15	–	–
Black wattle* (Grahamstown)	0·3	1·0	–	–	0·4	–	–
Green wattle (*A. decurrens*)	0·35	0·95	–	–	0·35	–	–
Silver wattle† (*A. dealbata*)	Trace	0·6	0·1	–	0·05	Trace	–
Silver wattle†	0·1	0·6	0·5	–	Trace	Trace	–
Golden wattle (*A. pycnantha*)	0·05	0·65	0·45	0·5	0·4	0·2	0·3

* Black wattle from Natal is that used for commercial afforestation; samples from the Albany district (Grahamstown) are of unknown botanical origin but possess all the botanical characteristics of *A. mearnsii*.
† Silver wattles have barks both 'rich' and 'poor' in (−)-*epi*catechin. In those examined the latter type predominates.

ethanol 207·5 and 281 mμ (ε at 281 mμ 3886) (Found: C, 58·9; H, 5·1; loss at 110°, 4·6. Calc. for $C_{15}H_{14}O_6,H_2O$: C, 58·4; H, 5·2; H_2O, 5·1%). $[\alpha]_D^{21}$ −57·1±0·7° in acetone–water (1:1) (*c*, 0·6).

(*d*) (+)-Gallocatechin. The crystals darkened rapidly after recrystallization from water, m.p. and mixed m.p. with (+)-gallocatechin from dried green-tea leaves (Bradfield, Penney & Wright, 1947; Bradfield & Penney, 1948) 185°. $\lambda_{max.}$ in ethanol 270·5 mμ (ε 1760). Gallocatechin from silver wattle gave an ochre colour with bis-diazotized benzidine spray and a blue with ferric alum spray. The gallocatechin also migrates to the identical position with (+)-gallocatechin on two-way chromatograms with solvent systems which separate catechin stereoisomers (Roberts & Wood, 1953).

(*e*) (−)-*epi*Gallocatechin. Ethyl acetate-soluble silver-wattle-bark extracts from which tannins were partly removed by passing the aqueous solution through a column of hide powder showed the presence of traces of a substance migrating to the same position on two-way chromatograms (Roberts & Wood, 1953) and giving the same colour reactions as (−)-*epi*gallocatechin.

Acacia pycnantha. A two-dimensional chromatogram of the ethyl acetate extract of golden-wattle bark showed the presence of a number of phloroglucinol-containing substances [amber colour with bis-diazotized benzidine (Roux & Maihs, 1960*b*)] in a pattern identical with that found in the extractives of dried green tea. The known catechins of dried green tea (Bradfield *et al.* 1947; Bradfield & Penney, 1948; Bradfield & Bate-Smith, 1950; Roberts & Wood, 1951, 1953) were isolated for comparison by the same methods described below for golden wattle, but without preliminary chloroform precipitation.

The ethyl acetate extract (230 g.) of golden-wattle bark was redissolved in ethyl acetate and the tannins were precipitated with chloroform as for silver wattle. The resulting soluble low-molecular-weight fraction (32 g.) was separated on 80 sheets of Whatman no. 1 paper with 2% acetic acid. On the dried sheets the bands were located by spraying test strips with the benzidine reagent and by ultraviolet light. Bands corresponding to R_F values 0·56 (0·4 g.), 0·50 (2·0 g.), 0·42 (2·2 g.), 0·35 (2·8 g.) and 0·29 (4·3 g.) were cut and eluted in 70% ethanol to give the weights of solids indicated. The fractions contained the following main components: R_F 0·56 [(−)-robinetinidol]; R_F 0·50 [(+)-catechin, gallic acid, (+)-gallocatechin]; R_F 0·42 [(−)-*epi*catechin, (−)-*epi*catechin gallate, (−)-*epi*gallocatechin]; R_F 0·35 [(−)-*epi*catechin gallate, (−)-*epi*gallocatechin gallate, (−)-*epi*gallocatechin]; R_F 0·29 [(−)-*epi*gallocatechin gallate]. Each band was separated on washed paper sheets in a partitioning mixture as described for silver wattle. The following substances were isolated after recrystallization from water, and where necessary compared with similar substances obtained from tea by identical methods, and also from other sources.

(*a*) (−)-Robinetinidol. This compound formed less than 20% of the fraction, R_F 0·56 in 2% acetic acid. Yield, 32 mg. M.p. and mixed m.p. with (−)-robinetinidol 203–204°. $\lambda_{max.}$ in ethanol 282 mμ (ε 3944). The compound gives the same colour reactions (blue with ferric alum, and yellow with bis-diazotized benzidine spray reagents) as (−)-robinetinidol, and their optical identity was shown by their concurrence on two-way chromatograms with the solvent systems below: R_F 0·78 in butan-1-ol–acetic acid–water (6:1:2) (solvent 1) and R_F 0·42 in 2% acetic acid (solvent 2) on Whatman no. 1 paper.

(*b*) (+)-Catechin. Yield, 700 mg. R_F 0·76 (solvent 1) and R_F 0·35 (solvent 2). M.p. and mixed m.p. with (+)-catechin from *Uncaria gambir* 177°. $[\alpha]_D^{21}$ +16·8±0·4° in acetone–water (1:1) (*c*. 2·3). $\lambda_{max.}$ in ethanol 281 mμ (ε 4080) (Found: C, 58·3; H, 5·4. Calc. for $C_{15}H_{14}O_6,H_2O$: C, 58·4; H, 5·2%). The acetate, m.p. and mixed m.p. with penta-acetyl-(+)-catechin 131°. $[\alpha]_D^{21}$ +28·4±0·4° in acetone (*c*, 0·8).

(*c*) (−)-*epi*Catechin. Yield, 80 mg. R_F 0·62 (solvent 1) and R_F 0·30 (solvent 2). M.p. and mixed m.p. with (−)-*epi*catechin from cocoa beans (Forsyth, 1955) 236° (decomp.). $[\alpha]_D^{20}$ −63·2±0·5° in ethanol (*c*, 0·4), and −57·4±0·5° in acetone–water (1:1) (*c*, 0·6). $\lambda_{max.}$ in ethanol 280 mμ (ε 4008) (Found: C, 56·4; H, 5·2. Calc. for $C_{15}H_{14}O_6,1{\cdot}5H_2O$: C, 56·8; H, 5·4%. Loss at 110° not determined). Evaporation of aqueous solutions of (−)-*epi*catechin during purification always resulted in some transformation into (−)-catechin, an epimerization reaction which occurs readily (Roberts & Wood, 1951, 1953).

(*d*) (−)-*epi*Catechin gallate. Yield, 135 mg. Initially this substance [and also (−)-*epi*gallocatechin gallate] crystallized with great difficulty, but crystallization was induced by slow evaporation of the solvent in a desiccator over $CaCl_2$ at room temperature. R_F 0·90 (solvent 1) and R_F 0·25 (solvent 2). M.p. 257–258°, and mixed m.p. with (−)-*epi*catechin gallate (m.p. 254–255°) from dried green-tea leaves 254–255°. $[\alpha]_D^{21}$ −181·0±1·0° in ethanol (*c*, 0·3), and −222·2±3·0° in acetone–water (1:1) (*c*, 0·3). $\lambda_{max.}$ in ethanol 280 mμ (ε 13570) (Found: C, 59·0; H, 4·3; loss at 110°, 2·1. Calc. for $C_{22}H_{18}O_{10},0{\cdot}5H_2O$: C, 58·5; H, 4·2; H_2O, 2·0%). The acetate (118 mg.) was formed from the *epi*catechin (110 mg.) by acetylation with the acetic anhydride–pyridine mixture, and the product recrystallized from 90% acetic acid, m.p. 118·5°. $[\alpha]_D^{21}$ −86·7±0·3° in acetone (*c*, 0·76).

(*e*) (+)-Gallocatechin. Yield, 410 mg. R_F 0·52 (solvent 1) and R_F 0·31 (solvent 2). M.p. 182–183°, and mixed m.p. with (+)-gallocatechin from *Casuarina equisetifolia* (Roux, 1957) 181–182°. $[\alpha]_D^{21}$ +10·1±0·3° in acetone–water (1:1) (*c*, 1·3). $\lambda_{max.}$ in ethanol 270·5 mμ (ε 1,820) (Found: C, 52·2; H, 4·8; loss at 110°, 8·0. Calc. for $C_{15}H_{14}O_7,1{\cdot}5H_2O$: C, 52·6; H, 5·3; H_2O, 10·5%). The gallocatechin (120 mg.) was acetylated as described above to give an acetate (116 mg.) recrystallized from ethanol, m.p. 141°. $[\alpha]_D^{21}$ +28·6±0·4° in acetone (*c*, 0·6).

(*f*) (−)-*epi*Gallocatechin. Yield, 55 mg. R_F 0·45 (solvent 1) and R_F 0·24 (solvent 2). M.p. 215°, and mixed m.p. with (−)-*epi*gallocatechin (m.p. 214–215°) from tea 215°. $[\alpha]_D^{21}$ −58·7±0·5° in ethanol (*c*, 0·5), and −65·0±1·4° in acetone–water (1:1) (*c*, 0·5). $\lambda_{max.}$ in ethanol 270·5 mμ (ε 1786) (Found: C, 53·8; H, 5·0; loss at 110°, 7·5. Calc. for $C_{15}H_{14}O_7,1{\cdot}5H_2O$: C, 54·1; H, 5·1; H_2O, 8·1%). The *epi*-gallocatechin from green tea (118 mg.) was acetylated to give an acetate (128 mg.), which was recrystallized from ethanol, m.p. 195°. $[\alpha]_D^{21}$ −16·4±0·3° in acetone (*c*, 0·7).

(*g*) (−)-*epi*Gallocatechin gallate. Yield, 150 mg. R_F 0·72 (solvent 1) and R_F 0·22 (solvent 2). M.p. 220°, and mixed m.p. with (−)-*epi*gallocatechin gallate (m.p. 220°) from tea 219–220° with decomposition at 221°. $[\alpha]_D^{21}$ −177±0·7° in ethanol (*c*, 0·3) and −220·5±1·6° in acetone–water (1:1) (*c*, 0·2). $\lambda_{max.}$ in ethanol 276 and 279·5 mμ (ε 9930 and 9950 respectively) (Found: C, 55·2; H, 4·8; loss at 110°, 4·9.

Calc. for $C_{22}H_{18}O_{11}.H_2O$: C, 55·5; H, 4·2; H_2O, 3·8%). The *epi*gallocatechin gallate (118 mg.) was acetylated as described above and the acetate (95 mg) recrystallized from 90% acetic acid, m.p. 119·5–120·5°.

(*h*) Gallic acid. Yield, 150 mg. M.p. and mixed m.p. with authentic gallic acid 250°. R_F 0·70 (solvent 1) and R_F 0·35 (solvent 2).

The values obtained for (−)-robinetinidol may be compared with those reported by Roux & Maihs (1960*a*), and values for the catechins, gallocatechins and their gallates with those by Vuataz, Brandenberger & Egli (1959), Bradfield *et al.* (1947), Bradfield & Penney (1948), Bradfield & Bate-Smith (1950), Mayer & Bauni (1957) and Roux (1957).

Estimation of catechins in wattle barks

Mature barks of black, silver, green and golden wattle were exhaustively extracted with methanol (six extractions of powdered bark of 24 hr. each at room temperature). Each extract was spotted on two-way chromatograms, with 4 mg. when finally spraying with the benzidine reagent and 6 mg. with the silver nitrate reagent. The catechins were estimated by the densitometric method as described by Roux & Maihs (1960*a*, *b*). The variation of catechin components within species and in black × green wattle hybrids is shown in Table 2, and their concentration in the four different wattles is shown in Table 3.

DISCUSSION

The close interrelationship of *A. mearnsii*, *A. decurrens* and *A. dealbata* compared with the more distantly related *A. pycnantha* is evident from a cursory examination of their leaves. The first three may again be differentiated into the pair *A. mearnsii* and *A. decurrens*, which were at one time regarded as varietal forms of the same species (*A. decurrens*), and *A. dealbata* which, although similar, is further removed from the *A. decurrens* group. Comparison of known chemical components in the phylogenetically old and relatively unspecialized organs, e.g. heartwoods and barks of these interrelated *Acacia* spp., is of interest, especially in view of the extensive work by Erdtman (1959) on the 'chemical taxonomy' of coniferous plants.

The heartwoods of the four species each contain (+)-7:3′:4′-trihydroxyflavan-3:4-diol, (+)-fustin, (−)-fisetinidol, fisetin and polymeric leuco-fisetinidin tannins. The concentration of these heartwood components in the wood is low and often variable (Roux & Evelyn, 1960), apart from the existing radial gradations (Roux & Paulus, 1961*a*, *b*). Differences in the concentration of heartwood components in different species (Table 1) are therefore not regarded as significant. The presence of identical components in the wattle heartwoods apparently confirms the general interrelationship between the four species. In the heartwood of *A. pycnantha* there are traces of minor components, e.g. gallic acid, which are not present in the *A. dealbata*–*A. decurrens* group.

By comparison, wattle barks have high tannin contents (17–40% on air-dry weight), with a relatively consistent composition of catechin components and average molecular weight of tannins present in each species (Evelyn, 1956). In the barks of *A. mearnsii* and *A. decurrens* identical catechins, (−)-robinetinidol, (+)-catechin and (+)-gallocatechin, are present but the concentration of (+)-catechin and (+)-gallocatechin appears consistently lower (0·45–0·65% and 0·25–0·4% respectively) in black wattles than in green wattles (1·1–1·5% and 0·4–0·5% respectively). In the black × green wattle hybrids the concentrations of these catechins approximate to those in green wattle (Table 2). Black wattles growing on shale appear to have a higher concentration of (−)-robinetinidol in the bark than green wattles growing on the same type of soil (Table 2). In *A. dealbata* (silver wattle) bark, (−)-*epi*catechin and traces of (−)-*epi*gallocatechin appear as additional components, increasing the emphasis on the 'phloroglucinol series' of catechins present. (−)-Robinetinidol is present, but in much decreased concentration (Table 3). In *A. pycnantha* the presence of (−)-*epi*catechin, (−)-*epi*catechin gallate, (−)-*epi*gallocatechin and (−)-*epi*gallocatechin gallate places even greater emphasis on the catechins of the 'phloroglucinol series' (Table 3), whereas (−)-robinetinidol, characteristic component of these *Acacia* barks, is present in low concentration. The isolated occurrence of gallic acid both in the bark and heartwood of *A. pycnantha* is of interest.

Considering the four species in the order of their closeness to black wattle in botanical character in the sequence *A. mearnsii*, *A. decurrens*, *A. dealbata* and *A. pycnantha*, *A. pycnantha* has the greatest number and total concentration of catechins of the 'phloroglucinol series' and also the lowest concentration of the single catechin of the 'resorcinol series' [(−)-robinetinidol] in the bark (Table 3). *A. dealbata* has a much decreased number of phloroglucinol catechins present in the bark, with the resorcinol catechin still in low concentration (Table 3). *A. decurrens*, closest to black wattle, has the identical composition of known catechins in the bark, but with those of the 'phloroglucinol series' present in higher concentration (Table 2). Hence the concentration and distribution of bark components in these *Acacias* species agree with the accepted systematics.

The isolation of (+)-catechin, (−)-*epi*catechin, (−)-*epi*catechin gallate, (+)-gallocatechin, (−)-*epi*gallocatechin, (−)-*epi*gallocatechin gallate and gallic acid from the bark of *A. pycnantha* is of interest in view of the presence of the identical mixture of compounds in dried green-tea leaves (Bradfield *et al.* 1947; Bradfield & Penney, 1948; Bradfield & Bate-Smith, 1950). In golden wattle,

however, the catechins are present as minor components associated with high-molecular-weight tannins, whereas in tea leaves they form the major phenolic components.

SUMMARY

1. The distribution of flavonoid compounds in the heartwoods and barks of four wattles (*Acacia* spp.) has been studied.

2. (−)-Fisetinidol, (+)-fustin, (+)-7:3′:4′-trihydroxyflavan-3:4-diol, fisetin and polymeric leucofisetinidin tannins are common components to the heartwoods of *A. mearnsii* (*A. mollissima*) (black wattle), *A. decurrens* (green wattle), *A. dealbata* (silver wattle) and *A. pycnantha* (golden wattle).

3. The barks of *A. mearnsii* and *A. decurrens* contain (−)-robinetinidol, (+)-catechin and (+)-gallocatechin; *A. dealbata* contains these catechins together with (−)-*epi*catechin and (−)-*epi*gallocatechin, and *A. pycnantha* contains these together with (−)-*epi*catechin, (−)-*epi*catechin gallate, (−)-*epi*gallocatechin, (−)-*epi*gallocatechin gallate and gallic acid.

4. The possible significance of the relative distribution of heartwood and bark components to the taxonomy of these wattles is discussed.

Thanks are due to Miss M. Tindale, Royal Botanic Gardens, Sydney, Australia, for confirming the relationships between these *Acacia* spp.; to Mr S. P. Sherry and Professor H. Shaw, Wattle Research Institute, Pietermaritzburg, Natal, for authentic bark and heartwoods of *A. mearnsii*, *A. decurrens* and *A. dealbata*, and for black × green wattle hybrids; to the Director, Union Department of Forestry, Pretoria, for the wood and bark of *A. pycnantha*; and to Dr R. Child, Tea Research Institute of East Africa, Kericho, Kenya, for dried green tea. This work was financed by the annual grant of the South African Wattle Growers' Union to the Leather Industries Research Institute.

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Biochem. J. (1961) **78**, 839

A Method for the Identification of the Immediate Product of Decarboxylation Reactions

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(*Received* 12 *May* 1960)

Under most physiological conditions the state of an enzymic decarboxylation product as bicarbonate ion, carbonic acid or carbon dioxide is determined mainly by local conditions of pH and gas concentration. The fact that the immediate end product of a decarboxylation reaction may actually be any one of the three forms would be masked by its rapid conversion into a form demanded by the buffer and gas composition of the medium. This conversion is rapid in systems where carbonic anhydrase is present. It has therefore been difficult with conventional methods to determine the immediate reaction product in carbon dioxide-producing systems.

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Carbonic anhydrase can be used as a tool in studying such reactions, as was first suggested by Roughton and co-workers (Meldrum & Roughton, 1933; Ferguson & Roughton, 1934; Roughton, 1935). Its use in the Warburg manometer established that carbon dioxide was the end product of the urea–urease system. In this system the relation of urease to urea was such that total urea-splitting occurred in a very short time, producing an initial abrupt rise of carbon dioxide pressure and a gradual return to equilibrium. In the presence of carbonic anhydrase no such rise occurred. Krebs & Roughton (1948) used this technique with yeast pyruvic decarboxylase and demonstrated that carbon dioxide was the immediate end product. Conway & O'Malley (1953), by rapid addition of carbonic anhydrase from the side arm of a Warburg flask to a mixture of yeast carboxylase and pyruvate producing carbon dioxide at a steady rate, altered the rate of production of the gas in a mathematically predictable way depending on whether the product were carbonic acid or carbon dioxide. They concluded that the product obtained depended to a certain extent on the source and purity of the yeast carboxylase.

It has been presumed (Woodbury & Esplin, 1959; Koch & Woodbury, 1960) that carbonic acid was the primary product of brain-tissue decarboxylations and that the role of carbonic anhydrase in brain cells was catalysis of bicarbonate to carbon dioxide. To test this hypothesis and to study further the physiological effect of carbonic anhydrase and its inhibitors in the central nervous system (Maren & Robinson, 1960), we utilized Krebs & Roughton's and Conway & O'Malley's methods on brain tissue of mice to determine the dominant end product of brain decarboxylations. It became apparent that inherent in both these methods was the necessity of a more powerful decarboxylase preparation than could be obtained with concentrated brain homogenates or minces in conventional Warburg vessels. In addition, the fact that the tissue was consuming oxygen at the same time as it was producing carbon dioxide obscured the small deflections of pressure necessary to differentiate the end products. A method was needed which was not influenced by oxygen consumption and which was relatively independent of the magnitude of the rate of carbon dioxide production.

In this paper a new technique is described that permits differentiation of these end products in physiological or other complex systems producing carbon dioxide at slow rates. The technique introduces a means of removing carbon dioxide from the medium by bubbling carbon dioxide-free air through the reactants. Carbon dioxide is thus removed as it is formed either primarily from the decarboxylation of substrate, or secondarily from the dehydration of carbonic acid as it is formed from substrate. The rate of collection of carbon dioxide is measured. Conditions, particularly rate of air flow and buffer pH, are chosen in such a way that the products can be distinguished kinetically. The distinction will ultimately depend on the velocity constants of several simultaneous reactions which are described in the theoretical section.

If carbon dioxide were the primary product, increase of flow rate will increase the rate of liberation of gas. Addition of carbonic anhydrase will decrease the rate of liberation of carbon dioxide. If carbonic acid or bicarbonate were the primary product, the rate of liberation of the gas will be relatively unaffected by flow rate but strongly increased by the addition of carbonic anhydrase. In this paper the method will be described and its application to the yeast pyruvic-decarboxylase system presented as a model.

MATERIALS AND METHODS

Apparatus. (See Fig. 1.) The reaction chamber consisted of a Toribara Ultra Filtration tube (obtained from Will Corp., Atlanta, Georgia) through which air previously passed through a soda-lime cannister and moistened by bubbling through distilled water was allowed to flow at a constant rate. Air was dispersed by the sintered-glass filter into minute bubbles, flowed through the reactants (usually enzyme, substrate and buffer) and was collected in a gas washing bottle (collecting vessel) containing a measured amount of NaOH in CO_2-free water. Phenolphthalein was added to the collecting solution, and the time for the indicator to reach a faint-pink end point was measured. Solutions could be added rapidly to the reaction vessel during constant air flow through a 15-gauge blunt syringe

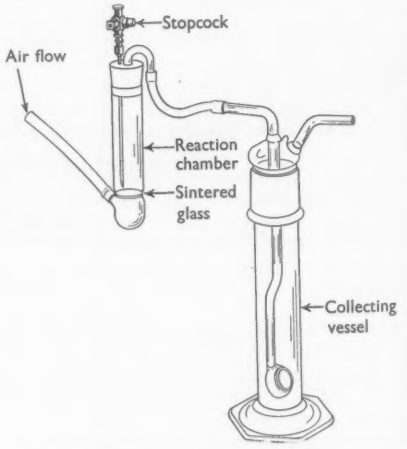

Fig. 1. Reaction chamber and collecting vessel. For explanation see text.

needle inserted through the rubber stopper. The needle was attached to a two-way metal stopcock, and syringes containing the solutions were connected to the cock. A typical experiment began with an accurately-measured constant flow of air passing through the empty reaction chamber and collected in the vessel containing 100 ml. of CO_2-free water and a few drops of phenolphthalein. The collecting vessel containing the base and indicator was found to be 99–100% efficient in collecting all CO_2 under the most rapid flow rates employed. Two syringes were attached to the stopcock, one containing 1 ml. of 0·2 M-sodium pyruvate and 1·0 ml. of 0·5 M-phosphate buffer, pH 7·8, and the other containing 0·5 ml. of yeast carboxylase of known activity. A few seconds before addition of the reactants to the reaction chamber, 0·5 ml. of 0·01 N-NaOH was added to the collecting vessel, thereby turning the phenolphthalein pink. At zero time the stopcock was opened, the syringes were emptied into the reaction vessel, and the time to the phenolphthalein end point in the collecting vessel was recorded. When carbonic anhydrase was used, it was included in the syringe containing the carboxylase preparation.

Yeast. Freshly pressed brewer's yeast, obtained from Schwarz Laboratories, Mount Vernon, New York, was spread in thin layers and allowed to dry at room temperature with the aid of a fan and then stored in the refrigerator until use. A portion (20 g.) of this dried preparation was incubated with 60 ml. of water for 2 hr. at 25° and then centrifuged, and the clear supernatant used as a source of carboxylase. The supernatant was then further diluted with water to give an activity as measured in the Warburg at 25° of 1–2 μmoles of CO_2/min. with a standard pyruvate solution.

Pyruvate. Sodium pyruvate, supplied by Schwarz Laboratories, Mount Vernon, New York, was prepared in an 0·2 M-concentration with CO_2-free distilled water. A portion (1 ml.) of this solution added to 0·5 ml. of diluted yeast carboxylase in 2 ml. of phosphate buffer yielded a steady rate of CO_2 production over a 10 min. period.

Carbonic anhydrase. A freeze-dried preparation of the chloroform–ethanol extract prepared from human blood by the method of Meldrum & Roughton (1933) containing 37 enzyme units (e.u.)/mg. was used. Enzyme activity was determined by the method of Maren, Ash & Bailey (1954). One unit by this method is roughly equivalent to 5 Meldrum–Roughton units. In control experiments with carbonic anhydrase, acetazolamide was added to the enzyme preparation in a concentration sufficient to totally inhibit the enzyme. Acetazolamide had no effect on the action of yeast carboxylase in the standard manometric procedure.

Results would be seriously affected if any of the solutions were contaminated with CO_2 or HCO_3^-. It was therefore extremely important to shake and then store all reactants under a vacuum and to perform control experiments on all solutions to ensure that the reactants were not contaminated with bicarbonate or other volatile acids.

Air flow. At flows greater than 60 ml./min., a calibrated flow meter (Flowmizer S/N 23976, tube size 2-15-3; Brooks Rotometer Co., Lansdale, Pa., U.S.A.) was used, accurate to approximately 2%. At lower flows, where meters are less accurate, flow was determined by counting the number of bubbles of air per minute flowing through the water bottle before entering the reaction vessel. By counting the number of bubbles and simultaneously collecting the gas in an inverted water-filled graduated cylinder it was possible to measure flow quite reproducibly and accurately.

Warburg manometer. For estimation of yeast carboxylase activity, Warburg shaking and constant-temperature apparatus manufactured by Gilson Medical Electronics, Middleton, Wis., U.S.A., was used. Calibrated conical flasks of approximately 20 ml. capacity with double side arms were employed.

Corrections were made for buffer effects by addition of HCl from the second side arm as described by Umbreit, Burris & Stauffer (1957).

THEORETICAL

The following scheme describes the events which may take place in the reaction and collecting vessels:

$$\begin{array}{ccccccc} CO_2 + OH^- + \text{phenolphthalein} & \rightarrow & HCO_3^- + \text{leucophenolphthalein} & & & & \\ \uparrow k_{app.} & & & & & & \\ CO_2 & \underset{k_{H_2CO_3}}{\overset{k_{CO_2}}{\rightleftharpoons}} & H_2CO_3 & \overset{K_{H_2CO_3}}{\rightleftharpoons} & H^+ + HCO_3^- & & (1) \\ \uparrow & & & & & & \\ X & & & & Z & & \end{array}$$

where X and Z are the rates of formation of CO_2 and HCO_3^- (or H_2CO_3) respectively in μmoles/min.

If CO_2 is the immediate product it can conceivably follow two pathways. It can be blown off by the air which is flowing through the reactants at a rate Y measured in ml./min. Such a rate of removal is given by

$$\frac{d[CO_2]}{dt} = -k_{app.} Y[CO_2] \qquad (2)$$

where $k_{app.}$ is a constant for the apparatus, including the diffusion constant of CO_2, a constant for bubble size and distribution, turbulence of liquid and air and volume of the reaction vessel and collecting tubing. This overall constant can be obtained by the addition of 10·0 μequiv. of $NaHCO_3$ to strong acid in the reaction vessel and measuring the length of time (t) required to neutralize 5·0 μequiv. of NaOH in the collecting bottle as various rates of flow. Then:

$$k_{app.} = \frac{2{\cdot}3 \log 2}{t_{\frac{1}{2}} . Y} = 0{\cdot}03 \text{ ml.}^{-1} \qquad (3)$$

for the flow rates used.

The second pathway CO_2 may take is that of hydration and secondary dehydration. From the scheme we may write the rate of change of CO_2 as:

$$\frac{d[CO_2]}{dt} = \frac{X}{V} - k_{app.} \cdot Y[CO_2] - k_{CO_2}[CO_2] + k_{H_2CO_3}[H_2CO_3] \quad (4)$$

where V is the volume of the solution in the reaction vessel.

The hydration and dehydration reactions have been extensively and accurately studied. Rate constants are given and methods reviewed by Roughton (1943) and Kern (1960). At 25° k_{CO_2} is 0·037 sec.$^{-1}$ and $k_{H_2CO_3}$ is 20 sec.$^{-1}$ (from the review by Kern, 1960).

Since H_2CO_3 or HCO_3^- is instantly interconverted (Roughton, 1941) we may write the sum of the rates of change of these two species as:

$$\frac{d[HCO_3^-]}{dt} + \frac{d[H_2CO_3]}{dt} = \frac{Z}{V} + k_{CO_2}[CO_2] - k_{H_2CO_3}[H_2CO_3]. \quad (5)$$

The equilibrium constant of the ionization is written as:

$$K_{H_2CO_3} = \frac{[H^+][HCO_3^-]}{[H_2CO_3]}. \quad (6)$$

The values for the equilibrium constant $K_{H_2CO_3}$ are reviewed by Kern (1960). The value of $1{\cdot}8 \times 10^{-4}$ is taken from the work of Scheurer, Brownell & LuValle (1958).

Since the hydrogen-ion concentration is maintained by the buffer, the rate of change of H_2CO_3 is proportional to the rate of change of HCO_3^-. From equation (6):

$$\frac{d[HCO_3^-]}{dt} = \frac{K_{H_2CO_3}}{[H^+]} \cdot \frac{d[H_2CO_3]}{dt}. \quad (7)$$

Inserting this into equation (5), we obtain:

$$\left(1 + \frac{K_{H_2CO_3}}{[H^+]}\right) \cdot \frac{d[H_2CO_3]}{dt} = \frac{Z}{V} + k_{CO_2}[CO_2] - k_{H_2CO_3}[H_2CO_3]. \quad (8)$$

A solution to the two simultaneous first-order differential equations (4) and (8) is given in the Appendix.

Inspection of equations (4) and (8) shows that if CO_2 is the primary product, at high flows ($k_{app.} \cdot Y$ very large) CO_2 will be quantitatively removed; the rate-limiting factor is its production (X). If, on the other hand, H_2CO_3 or HCO_3^- is the primary product, at high flows the removal of formed CO_2 is limited by the rate of the dehydration reaction. Approximate equations applicable to the conditions employed here are:

(i) For the hypothetical case of CO_2 production in terms of total CO_2 liberated (R). Let

$$m = 1 + \frac{K_{H_2CO_3}}{[H^+]}.$$

$$R = X\left[\frac{k_{app.} \cdot Y}{k_{CO_2} + \frac{k_{H_2CO_3}}{m}}\right]\left[\frac{-1}{\frac{k_{H_2CO_3}}{m} + k_{CO_2} + k_{app.} \cdot Y} + t\right] \quad (9)$$

(ii) For the hypothetical production of H_2CO_3 or HCO_3^-.

$$R = Z\left[t - \left\{1 + \frac{k_{CO_2}}{\left(\frac{k_{H_2CO_3}}{k_{app.} \cdot Y}\right)} \cdot m\right\} \times \left\{1 - \exp\left(\frac{-k_{app.} \cdot Y \cdot t}{1 + \frac{k_{CO_2}}{k_{H_2CO_3}} \cdot m}\right)\right\}\right] \quad (10)$$

RESULTS

Experiments with yeast carboxylase

Two syringes were attached to the stopcock, one containing 0·5 ml. of diluted yeast carboxylase and 2 ml. of 0·5 M-phosphate buffer, pH 7·8, and the other containing 1 ml. of 0·2 M-sodium pyruvate. For each series of experiments the activity of the yeast extract was measured in the Warburg. Production of CO_2 (X) varied from 0·8 to 2·6 μmoles/min. The collecting vessel contained 5 μmoles of NaOH in 100 ml. of water. The term R is then equivalent to the amount of NaOH added minus the amount required to bring the CO_2-free water to a faint-pink end point (usually 1·5 μmoles/100 ml.). The flow rate was varied from 10 to 60 ml./min. The solutions were injected and the time to the phenolphthalein end point was measured. The results of several experiments are given on Table 1, and compared with the predicted values for primary CO_2 formation obtained by substitution in equation (9.) A representative experiment is plotted in Fig. 2.

Experiments with bicarbonate

The hypothetical situation in which a substrate-enzyme system produces HCO_3^- or H_2CO_3 was simulated by the slow constant addition of $NaHCO_3$ solution to phosphate buffer under conditions of constant air flow. A syringe filled with 0·03 M-$NaHCO_3$, whose plunger was slowly depressed at a constant rate by a micrometer device attached to a low-speed motor, was attached to the stopcock on the reaction flask. The rate of addition was adjusted by a rheostat which controlled the motor. With a constant flow of air through 3·5 ml. of 0·5 M-phosphate buffer in the reaction chamber, the

Table 1. *Reaction times at various rates of air flow and various rates of yeast pyruvic-decarboxylase activity*

The collecting vessel contains, in all cases, 3·5 μmoles of phenolphthalein-titratable NaOH (*R*). Predicted values are obtained from substitution in equation (9).

Carboxylase activity CO_2 production (*X*) (μmoles/min.)	Rate of air flow (*Y*) (ml./min.)	Experimental reaction times (*t*) (min.)		Predicted reaction time (min.)	*R*/*X* (min.)
0·8	22	10·7	11·0	12·8	4·35
	49	6·2	6·0	5·8	—
	61	4·8	4·9	4·7	—
1·06	24	7·6	7·4	8·9	3·3
	37	4·1		3·4	—
	51	3·5	3·4	3·3	—
1·87	10	10·8	11·0	12·2	1·9
	15	6·9	7·4	8·3	—
	27	3·9	4·2	4·7	—
	40	3·0	2·9	3·2	—
	48	2·5	2·7	2·7	—
	60	2·0	2·1	2·1	—
2·85	12	6·1	6·3	6·9	1·2
	26	3·0	3·2	3·3	—
	42	2·0	2·2	2·1	—

Table 2. *Reaction times at various rates of air flow and various rates of addition of 0·03 M-sodium hydrogen carbonate to phosphate buffer,* pH 7·8, *in the reaction chamber*

The collecting bottle contains 3·5 μmoles of phenolphthalein-titratable NaOH (*R*). Theoretical times are calculated by substitution in equation 10 of the text.

Rate of addition of 0·03 M-$NaHCO_3$ (*Z*) (μmoles/min.)	Rate of air flow (*Y*) (ml./min.)	Experimental reaction times (*t*) (min.)		Theoretical reaction times (min.)
1·20	23	13·8	13·9	14·3
	30	13·9	13·8	13·8
1·87	18	12·0	12·1	13·0
	23	12·1	12·2	11·1

motor was started and the time required to reach the phenolphthalein end point in the collecting vessel was measured. Flow was varied from 10 to 60 ml./min. Results at different rates of addition are given in Table 2 and compared with the predicted values for primary HCO_3^- formation from equation (10). A representative experiment is plotted in Fig. 2.

Experiments with carbonic anhydrase

In these experiments the flow rate was held constant at 30 ml./min.; 0·2 ml. of carbonic anhydrase solution containing 370 e.u./ml. was mixed with the yeast extract; with this exception the technique was the same as that previously described for the carboxylase system. In experiments with constant flow of $NaHCO_3$, carbonic anhydrase was first added to the phosphate buffer. As a control the same volume of enzyme solution plus 0·1 ml. of 1 mg./ml. acetazolamide was added. Results are given in Table 3. It is evident that carbonic anhydrase prolongs the reaction time in the yeast carboxylase system and shortens it in the experiments with $NaHCO_3$.

DISCUSSION

Results show that it is possible to distinguish between the two end products, as predicted by the theoretical treatment. Comparison of the experimental curves in Fig. 2 shows that it is principally the rate of air flow that enables the differences in the reaction velocities to become apparent. Velocities principally concerned are the terms $k_{app.}.Y$ contrasted with $k_{H_2CO_3}\frac{[H^+]}{K_{H_2CO_3}}$. If $k_{app.}.Y$ is sufficiently large, the amount of CO_2(*R*) which is blown off is equal to $X.t$. On the other hand, in the HCO_3^- experiments the term $k_{app.}.Y$ plays no role at flow rates beyond a point where it is permissible to neglect the reverse reaction. The rate of liberation of CO_2 $\left(\frac{R}{t}\right)$ is determined principally by $k_{H_2CO_3}.\frac{[H^+]}{K_{H_2CO_3}}$.

The two model systems of Fig. 2 are arranged so that the rates of HCO_3^- addition on the one hand and CO_2 formation on the other are constant and equal. Regardless of the magnitude of these rates,

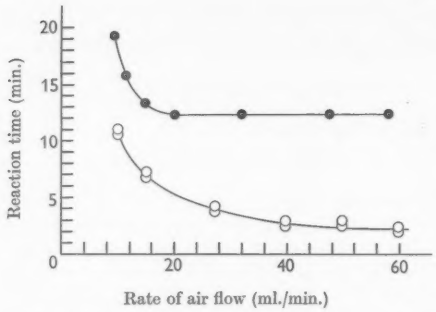

Fig. 2. Representative curves of reaction times versus rate of air flow in the yeast pyruvic-decarboxylase system (○) and in the $NaHCO_3$ addition to buffer experiments (●). X and Z = 1·87 μmoles/min. For details see text.

the differences between the two species will now be apparent at high flow. Confirmation is given by the use of carbonic anhydrase, which by increasing the magnitude of both k_{CO_2} and $k_{H_2CO_3}$ decreases the rate of liberation of CO_2 if CO_2 is the end product and increases it if HCO_3^- or H_2CO_3 is the end product (Table 3).

The effect of carbonic anhydrase on the reaction time in the $NaHCO_3$ experiments is much greater than in the CO_2 (yeast carboxylase) system. Equations (4) and (8) show that the effect on $NaHCO_3$ dehydration is exponential, and on CO_2 hydration is inversely proportional, accounting for the differences observed in Table 3.

Experiments with yeast pyruvic decarboxylase show unequivocally that CO_2 is the dominant, if not the sole, end product. The response to various rates of flow and to the addition of carbonic anhydrase is entirely consistent with this. This finding is somewhat in contrast with the results of Conway & O'Malley (1953), who concluded that, in the decarboxylation of keto acids by yeast carboxylase, CO_2 and H_2CO_3 (or HCO_3^-) are both primary products but that one or the other may predominate, depending on the total conditions. It is possible with the present method to make an analysis concerning the dominance of CO_2 as an end product, as follows: from Fig. 2 it is evident that at flow rates where the curve is asymptotic $t = R/X$. If a significant amount of HCO_3^- or H_2CO_3 were being formed in addition to CO_2 (Conway & O'Malley, 1953) this would not be the case, t at asymptote being greater than R/X. From these data, it appears that if any HCO_3^- or H_2CO_3 is formed in addition to CO_2, it is undetectable.

APPENDIX

Solutions to equations (4) and (8) in the text may be obtained for $[CO_2]$, $[H_2CO_3]$ and $[HCO_3^-]$ by the method of operators. These solutions are in the form $a+be^{-r_1t}+ce^{-r_2t}$, where a, b and c are different constants for the concentrations of the three substances. The rate constants of the two exponential terms are the two roots, $D = -r_1$ and $D = -r_2$, of the equation

$$m.D^2+(k_{H_2CO_3}+m.k_{app.}.Y+m.k_{CO_2})D + k_{app.}.Y.k_{H_2CO_3}=0 \quad (1)$$

where $m = 1+\dfrac{K_{H_2CO_3}}{[H^+]}$.

Equation (1) holds whether CO_2, H_2CO_3 or HCO_3^- is the primary product. Designating C as the sum of the concentrations of the three components, and C_∞ as the value of C at infinite time, the solution for C, if CO_2 is the primary product, may be written

$$C = C_\infty - \frac{C_\infty r_2 - \frac{X}{V}}{r_2-r_1}.e^{-r_1t} - \frac{\frac{X}{V}-C_\infty r_1}{r_2-r_1}.e^{-r_2t} \quad (2)$$

where C_∞ is given by

$$C_\infty = \frac{X}{V}\left(\frac{1}{k_{app.}.Y}\right)\left(1+\frac{k_{CO_2}}{k_{H_2CO_3}}.m\right). \quad (3)$$

Table 3. *Effect of carbonic anhydrase on the reaction times of the yeast-carboxylase system and the simulated bicarbonate- or carbonic acid-producing system at constant flow rate*

Carbonic anhydrase (74 e.u.) is added to the syringe containing yeast carboxylase and buffer. In experiments with $NaHCO_3$, carbonic anhydrase is added to the phosphate buffer in the reaction vessel. As a control, 0·1 ml. of 1·0 mg./ml. acetazolamide is added to the carbonic anhydrase solution. Air flow is constant at 30 ml./min. The collecting bottle contains 3·5 μmoles of phenolphthalein-titratable NaOH (R). Figures are means of five experiments ± S.E.M.

System	Rate of production of CO_2 or HCO_3^- (X or Z) (μmoles/min.)	Reaction times (min.)		
		No carbonic anhydrase	Carbonic anhydrase plus acetazolamide	Carbonic anhydrase alone
Yeast carboxylase	1·87 (X)	4·0±0·07	4·0±0·06	4·6±0·09
$NaHCO_3$	1·87 (Z)	12·0±0·03	12·1±0·04	4·8±0·15

P values <0·01 between experiments with and without carbonic anhydrase.

If HCO_3^- or H_2CO_3 is the primary product, Z replaces X in equation (2) above and C_∞ is given by

$$C_\infty = \frac{Z}{V}\left(\frac{1}{k_{app.}.Y}\right)\left(1+\frac{k_{app.}.Y+k_{CO_2}}{k_{H_2CO_3}}.m\right). \quad (4)$$

The amount of CO_2 removed, R, is given by

$$R = Xt - CV \quad (5)$$

when CO_2 is the primary product. When H_2CO_3 or HCO_3^- is the primary product

$$R = Zt - CV. \quad (6)$$

It is then possible to calculate precisely the amount of CO_2 blown off from either system in any circumstances. If the values of the hydration and dehydration velocity constants at 25° are substituted and $Y = 27{\cdot}5$ ml./min. is chosen, equation (1) above yields

$$r_1 = 0{\cdot}000462 \text{ sec.}^{-1} \text{ and } r_2 = 0{\cdot}52 \text{ sec.}^{-1}$$

at a pH of 7·80. Therefore the first exponential term in equation (2) above becomes relatively small only after 10^4 sec., whereas the second exponential term is negligible after 10 sec. For the times actually employed in these experiments the first exponential term of equation (2) may be expanded with retention of the first term of the series. The second exponential term of equation (2) may be dropped.

Certain limiting cases are of interest. If $k_{app.}.Y$ is large, we may neglect the first term in equation (1) above. If $k_{app.}.Y$ is small we may neglect the third term in equation (1). r_1 and r_2 are then given by simple expressions. Substitution of the values for r_1 and r_2 in equation (2) and solving equations (3) and (5) yield, for the case of CO_2 as the primary product,

$$R = X\left[1-\frac{\frac{k_{H_2CO_3}}{m}+k_{CO_2}}{k_{app.}.Y}\right]\left[\frac{-1}{k_{app.}.Y}+t\right] \quad (7)$$

when $k_{app.}.Y$ is large, and text equation (9) when $k_{app.}.Y$ is small. For the case of HCO_3^- or H_2CO_3 production, retention of only the first term of the expansion of the first exponential term in equation (2) is not sufficient. If the first exponential term of equation (2) is not expanded and the second exponential term is neglected we obtain

$$R = Z\left[t-\frac{m}{k_{H_2CO_3}}\left\{1-\exp\left(\frac{-k_{H_2CO_3}.t}{m}\right)\right\}\right] \quad (8)$$

for H_2CO_3 or HCO_3^- production when $k_{app.}.Y$ is large. Expansion of equation (8) and retention of only two terms results in

$$R = Z\frac{k_{H_2CO_3}.t^2}{m} \quad (9)$$

which is applicable for short reaction times. If $k_{app.}.Y$ is small, text equation (10) is obtained, which yields, on expansion to two terms,

$$R = Z\frac{k_{app.}.Y.t^2}{1+\frac{k_{CO_2}}{k_{H_2CO_3}}.m}. \quad (10)$$

SUMMARY

1. A method is proposed to distinguish between carbon dioxide and HCO_3^- ion as end products of decarboxylation reactions.

2. The method is dependent upon the removal of carbon dioxide by flowing air at various rates through reactants and then quantitatively collecting and measuring the liberated carbon dioxide. Theoretical and experimental evidence are presented, demonstrating that the distinction is dependent on the velocity constant of the dehydration of carbonic acid.

3. Experiments with yeast pyruvic carboxylase and the constant addition of sodium hydrogen carbonate, both with and without carbonic anhydrase, confirmed the validity of the assumptions.

4. Yeast pyruvic decarboxylase produces carbon dioxide as its dominant, if not sole, end-product.

The author wishes to thank Dr T. H. Maren for his support, enthusiasm and helpful suggestions. He is indebted to Dr A. L. Koch for his interest and advice, particularly in the development of the theoretical portion.

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Effects of Trauma on Incorporation of L-[^{35}S]Cysteine into Tissue Glutathione

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(*Received* 3 *August* 1960)

When mice, rats or rabbits are subjected to certain procedures which produce stress or injury, there is a fall in the concentration of non-protein-sulphydryl-group compounds of the liver and other tissues (Stolfi, 1936; Beck & Linkenheimer, 1952; Lindan & Work, 1953; Bartlett & Register, 1953; Beck & Rieck, 1958). The literature suggests that such decrease may be due mainly to decreased synthesis of glutathione, the most abundant component of liver non-protein-sulphydryl-group compounds. To test this possibility we have studied effects of tourniquet trauma on incorporation of the radioactivity of L-[^{35}S]cysteine into mouse-tissue glutathione.

METHODS

In each control or trauma experiment, pooled tissue and excreta samples were taken from 10 Carworth Farms Webster strain male mice. In the trauma experiments, the blood supply to both hind legs was occluded for 3 hr.; mice were killed 2·5 hr. after removal of the rubber-band ligatures. L-[^{35}S]Cystine, 5–20 μc/mg., was secured from Schwarz Bioresearch Inc., Mt. Vernon, New York, dissolved in 0·5 N-HCl and reduced electrolytically to L-[^{35}S]cysteine, in a model 1930 Desalter (Research Specialties Co., Berkeley, Calif., U.S.A.). A weighed amount, about 0·1 g. (about 1·5 μc) of radioactive L-cysteine solution was injected by tail vein into each mouse 0·75 or 1·5 hr. before killing. Pooled tissue samples were kept frozen on solid carbon dioxide until analysed. Material termed carcass consisted of everything left over after the tissue and excreta samples had been taken. For tissues other than liver, the following procedure was employed. The weighed sample was ground in a mortar with ice-cold 5% trichloroacetic acid, about 5 ml./g. The brei was filtered through Whatman no. 1 paper into a volumetric flask preselected to have a volume not less than 10 and not more than 20 ml./g. of sample. Trichloroacetic acid (5%) was poured into the paper funnel until the flask was full to the mark. Trichloroacetic acid was then removed from a portion of the filtrate by extraction with ice-cold, peroxide-free ether. Three successive extractions were made under nitrogen in a Thunberg tube, each with 2 vol. of ether. Residual ether was removed by evacuation. After removal of trichloroacetic acid, a copper glutathionate precipitate was secured by the method of Hopkins (1929), about 50-fold excess of carrier glutathione (GSH) being used. This precipitate was used to estimate the amount of radioactivity incorporated into GSH of the tissue sample. For each liver sample, the procedures of Waelsch & Rittenberg (1941) were employed to secure a 2 ml./g. extract in 10% trichloroacetic acid, and from this two copper glutathionate precipitates. One of the precipitates was secured with, the other without, carrier GSH, to permit estimation of liver GSH, by isotopic dilution (Kamen, 1947), as well as total radioactivity incorporated into liver GSH. Each precipitate remaining after the original extraction was re-extracted with 5% trichloroacetic acid, 20 ml./g., to permit estimation of non-protein-SH not extracted by use of 2 ml. of 10% trichloroacetic acid/g. This amounted to about 12–20% of total extracted non-protein-SH (cf. Table 2). Non-protein-SH in extracts was estimated essentially as described by Grunert & Phillips (1951). Losses in non-protein-SH from ether extraction ranged from zero (SH solutions, liver extracts) to 15%. Estimations of radioactivity were made, for both trichloroacetic acid-containing and ether-extracted extracts, by use of P611 counting mixture (Davidson & Feigelson, 1957), for copper glutathionate suspensions by use of thixcin counting gel (Nathan, Davidson, Waggoner & Berlin, 1958). The copper glutathionate was brought into suspension by ultrasonic bombardment. Counting rate for each particular sample-counting mixture (10 or 20 ml.) was obtained with a low-potassium five-dram counting vial (Wheaton Co.) and a Packard Co. Tri-Carb liquid-scintillation spectrometer. Quenching was corrected for by use of internal standards (Davidson & Feigelson, 1957). After allowing for quenching, corresponding trichloroacetic acid-containing and free extracts gave nearly identical counting rates; very little radioactivity was found in ether supernatants.

RESULTS

The data of Table 1 indicate that decreases in tissue non-protein-SH compounds were induced by trauma in all of the tissues examined, but most markedly in liver and kidney. Differences in liver non-protein-SH compounds between control and traumatized groups of mice were very similar to those found in earlier work (Beck & Linkenheimer, 1952; Beck & Rieck, 1958). In the earlier work, in which liver non-protein-SH was estimated for each mouse used, P values for control versus traumatized groups of mice were almost invariably less than 0·001; hence present differences are considered significant. The concentration ratios of Table 1 were calculated to simplify comparisons between control and trauma experiments, for distribution of administered ^{35}S into (*a*) GSH, and (*b*) all non-protein compounds:

$$\text{Concentration ratio} = \frac{\text{Counts/min./mg. of tissue used to obtain } ^{35}\text{S in (}a\text{) GSH or (}b\text{) all non-protein}}{\text{Counts/min. (injected material)/mg. of mouse}}.$$

From this definition it follows that if administered ^{35}S were uniformly distributed throughout the entire body and none was excreted, and if all were incorporated into GSH, all numbers of columns (*a*) and (*b*) of Table 1 would be 1·0. The concentration ratios obtained for tissue GSH [cf. rows (*a*) of Table 1] indicate that L-[^{35}S]cysteine incorporation into GSH was: (1) much greater for liver and kidney than for the other tissues examined; (2) greater for tissues of control than of traumatized mice, especially for liver and kidney; (3) greater for a 0·75 hr. than for a 1·5 hr. incorporation time. This last finding suggests that GSH is an early intermediate in the metabolism of L-cysteine.

The concentration ratios obtained for ^{35}S in all non-protein compounds [cf. rows (*b*), Table 1] indicate that liver and kidney of both control and traumatized mice selectively accumulated L-cysteine or derivative non-protein compounds or both, and to an extent well beyond what would have been expected if the accumulation had been due mainly to GSH synthesis. If, in corresponding control and trauma experiments, the ratio

$$\frac{^{35}S \text{ in L-cysteine}}{^{35}S \text{ in all non-protein compounds}}$$

were the same for corresponding tissues, then the (*b*) values of Table 1 would indicate what fraction of administered L-[^{35}S]cysteine had been available for synthesis of labelled GSH, and (*a*)/(*b*) ratios would indicate how efficient the tissues had been in carrying out such synthesis. From Table 1 it is apparent that all liver and kidney (*a*)/(*b*) ratios were much lower in trauma experiments than in corresponding control experiments, a finding which strongly suggests that these tissues of traumatized mice were less efficient in GSH synthesis than were corresponding tissues of control mice. The relatively high (*b*) values found for tissues of traumatized mice, and the greater excretion of ^{35}S found in control than in corresponding trauma experiments, indicate that trauma-induced decrease in tissue synthesis of labelled GSH was not due mainly to trauma-induced decrease in availability of L-[^{35}S]cysteine to the tissues. Skeletal-muscle samples were taken from the hind legs. Therefore it is likely that the much higher (*b*) values found for muscle in the trauma experiments were due to accumulation of oedema fluid in the hind legs, as a result of the trauma.

In five of the experiments, liver GSH was estimated by isotopic dilution (Kamen, 1947). The data obtained (Table 2) indicate that trauma-induced decrease in liver GSH was of a magnitude sufficient to account for the greater part of trauma-induced decrease in liver non-protein-SH compounds.

Table 1. *Effects of trauma on tissue non-protein-sulphydryl-group compounds and on distribution of* L-[^{35}S]*cysteine into* (a) *glutathione and* (b) *all non-protein compounds*

NPSH, Tissue non-protein SH (m-equiv. of S/kg.); (*a*) and (*b*), tissue-concentration ratios for GSH and all non-protein compounds respectively. For definition of concentration ratio, see text. Time between intravenous injection of L-[^{35}S]cysteine and killing, 1·5 or 0·75 hr. C_1, etc., control experiments; T_1, etc., trauma experiments; A_1, experiments in which mice had been adrenalectomized 2 days earlier. Muscle samples were obtained from hind legs, and therefore, in trauma experiments, were subjected to prior occlusion of circulation.

Time interval	1·5 hr.						0·75 hr.		
Estimation made	C_1	C_2	C_3	C_4	T_1	T_2	C_5	T_3	A_1
Liver NPSH	5·80	6·84	6·38	6·56	3·32	3·29	7·22	2·70	7·55
(*a*)	0·28	0·24	0·27	0·36	0·13	0·13	0·62	0·17	0·83
(*b*)	1·35	1·39	1·10	1·18	1·34	1·14	1·98	0·98	2·29
Kidney NPSH	4·33	5·11	4·69	3·52	2·93	1·92	4·23	2·28	3·58
(*a*)	0·47	0·22	0·30	0·25	0·08	0·19	0·59	0·19	0·49
(*b*)	1·55	1·39	1·33	1·79	1·73	2·58	1·60	2·10	1·08
Mucosa NPSH	3·48	3·29	2·60	2·93	2·80	2·12	3·16	2·41	3·16
(*a*)	0·12	0·15	0·06	0·10	0·06	0·07	0·20	0·09	0·16
(*b*)	0·66	0·69	0·52	0·72	0·54	0·36	0·56	0·39	0·43
Carcass NPSH	—	—	—	—	—	—	1·11	0·65	1·11
(*a*)	0·041	0·026	0·032	0·016	0·022	0·019	0·054	0·038	0·080
(*b*)	0·265	0·201	0·273	0·200	0·396	0·511	0·307	0·361	0·252
Muscle NPSH	0·81	0·99	0·59	0·39	0·39	0·16	0·59	0·39	0·49
(*a*)	—	0·010	0·013	0·011	0·009	0·020	0·034	0·021	—
(*b*)	0·104	0·110	0·080	0·078	0·270	0·473	0·010	0·285	—
S recovered in all non-protein compounds (%)	32	27	33	27	44	55	41	41	37
S recovered in excreta (%)	30	23	28	29	7	3	8	3	3

Table 2. *Effects of trauma on liver glutathione, estimated by isotopic dilution*

GSH and non-protein-SH-group compounds ($NPSH_1$, $NPSH_2$) were determined in portions from extract in 10% trichloroacetic acid, 2 ml./g. of liver. The precipitate from this first extract was re-extracted with 5% trichloroacetic acid, 20 ml./g. of liver. $NPSH_2$ was estimated in this second extract.

Time interval ...	1·5 hr.		0·75 hr.		
Factor estimated (m-equiv. of S/kg.)	C_4	T_2	C_5	T_3	A_1
GSH	4·86	2·59	4·73	2·28	4·57
$NPSH_1$	5·58	2·64	5·99	2·37	6·38
$NPSH_2$	0·98	0·65	1·23	0·33	1·17
$NPSH_1 + NPSH_2$	6·56	3·29	7·22	2·70	7·55

DISCUSSION

Experimentally, each particular tissue of control mice and rats exhibits, within a characteristic range, constancy in GSH concentration and in concentration of non-protein-sulphydryl-group compounds. Since the literature indicates that plasma GSH is very low, it is unlikely that passage of GSH through cell membranes is important in determining GSH concentrations of particular tissues. Rather, the GSH concentration existing in a particular tissue is probably determined mainly by a steady-state equilibrium peculiar to that tissue, in which (*a*) rate of synthesis and (*b*) rate of degradation of GSH in the tissue are nearly equal. A trauma-induced decrease in GSH of a particular tissue might be due mainly to decrease in (*a*) or increase in (*b*). If trauma reduced (*a*) to zero, no part of ^{35}S administered as L-cysteine would appear in the tissue GSH. Therefore the much smaller incorporation of L-[^{35}S]cysteine into tissue GSH of traumatized than that of control mice, noted in the present experiments, would appear to be in accord with the hypothesis that trauma-induced decrease in tissue GSH is due mainly to decreased synthesis rather than increased degradation of the compound. Decreased synthesis might be due to decreased efficiency of the GSH-synthesis mechanism or decreased availability of L-[^{35}S]-cysteine for incorporation into GSH or both, as would occur, for example, if trauma markedly increased oxidation of L-cysteine toward sulphate. The literature indicates that decreased efficiency of GSH-synthesis mechanisms may be expected to occur in association with trauma: (*a*) Bloch (1949) has shown that both adenosine triphosphate and oxygen are necessary for synthesis of GSH in a pigeon-liver preparation *in vitro*; (*b*) animals in traumatic shock exhibit tissue anoxia, especially in liver and kidney (Wiggers, 1950); (*c*) Le Page (1946) and McShan, Potter, Goldman & Shipley (1945) have shown that severe trauma or haemorrhage results in depletion in body tissues of adenosine triphosphate and other energy-rich phosphate compounds.

SUMMARY

1. In comparison with controls, mice whose blood supply to the hind legs had been occluded for 3 hr., and which were killed 2·5 hr. after removal of hind-leg ligatures, exhibited in their tissues, especially liver and kidney, appreciably lower values for both concentrations of non-protein-SH-group compounds and incorporation of L-[^{35}S]cysteine into glutathione.

2. This parallelism indicates that trauma induces decreases in tissue non-protein-SH-group compounds mainly by inhibiting synthesis of glutathione *in vivo*.

3. Most of the decrease in liver non-protein-SH-group compounds was accountable for by decrease in glutathione as estimated by isotopic dilution.

This research was supported by NIH Grant RG-3740. We are indebted to Dr Clyde Williams, Veterans Administration Hospital, Pittsburgh, for access to and advice in use of the Packard Co. Tri-Carb spectrometer.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **399th Meeting** *of the Society was held at the National Institute for Medical Research, Mill Hill, London, N.W. 7, on Saturday, 22 October* 1960, *when the following papers were read.*

COMMUNICATIONS

(1) The Structure of Urinary Oestriol Glucuronoside

By J. G. D. Carpenter and A. E. Kellie. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

Oestriol [oestra-1:3:5(10)-triene-3:16α:17β-triol] in the 'combined' form was first isolated from late pregnancy urine by Cohen & Marrian (1936). The C-3 hydroxyl group in the conjugate is free and it contains one glucosiduronic acid residue per molecule of steroid (Cohen, Marrian & Odell, 1936; Grant & Marrian, 1948, 1950). No evidence has been forthcoming, however, as to whether oestriol is conjugated at the C-16 or C-17 position.

In the present work, the two isomers methyl (3-methoxy-17β-acetoxyoestra-1:3:5(10)-trien-16α-yl 2:3:4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (I) and methyl (3-methoxy-16α-acetoxyoestra-1:3:5-(10)-trien-17β-yl 2:3:4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (II) were synthesized. First, 3-methoxy-16α-hydroxyoestra-1:3:5(10)-trien-17-one (III) was prepared using a procedure described by Leeds, Fukushima & Gallagher (1954) for synthesizing the phenol; (III) was then combined with methyl 2:3:4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate (IV) (Meystre & Miescher, 1944), to yield methyl (3-methoxy-17-oxo-oestra-1:3:5(10)-trien-16α-yl 2:3:4-tri-*O*-acetyl-β-D-glucopyranosid)uronate. Reduction of the C-17 ketone with sodium borohydride in methanol at 5° followed by acetylation afforded (I). Acetylation of (III), low-temperature reduction of the C-17 ketone and coupling with (IV) yielded (II).

The two isomers, (I) and (II), exhibited essentially the same infrared spectra in CS_2 and in KBr disks. They could not be completely resolved by chromatography in the Bush 'A' system (Bush, 1952) either on Celite 535 or on paper, or in a light petroleum–methanol system run at 2° (Edwards, 1960). The compounds had different melting points and crystalline forms; both had low negative optical rotations in ethanol.

Oestriol glucuronoside was extracted from late pregnancy urine with butanol (Cohen, 1950). The final extract was chromatographed first on alumina (Crépy, Jayle & Meslin, 1957) and then on Celite 535 in the system butanol–toluene–acetic acid–water (25:75:30:70, by vol.). The eluate from the Celite column was examined by paper chromatography (Lewbart & Schneider, 1955) and the Kober reaction (Ittrich, 1958). The residue from fractions containing oestriol glucuronoside was methylated and acetylated and then chromatographed in the Bush 'A' system on Celite 535; two peaks of Kober-positive substances were obtained. The solid from the second peak was homogeneous when chromatographed on paper (phosphomolybdate stain) and had an infrared spectrum identical with the synthetic isomers, (I) and (II). The melting point ranged over about 10°. This material was resolved into two components by fractional crystallization. Comparison of each component with (I) and (II) by mixed-melting-point determinations indicates that both ring D isomers of oestriol glucuronoside are present in pregnancy urine.

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Meystre, C. & Miescher, K. (1944). *Helv. chim. Acta*, **29**, 231.

(2) Biosynthesis of Squalene from [2-^{14}C]-Mevalonic Acid by a High-Speed Supernatant Fraction of Rat-Liver Homogenate

By L. Gosselin* and Estelle Podber-Wagner. (*Department of General Pathology, University of Liège, Belgium*)

A rat-liver homogenate prepared in phosphate buffer (Bucher & McGarrahan, 1956) was centrifuged at 105 000 *g* for 60 min. The supernatant

* Chargé de Recherches du F.N.R.S. (Belgium).

fraction was found to be capable of synthesizing only minute amounts of squalene. The activity of this fraction could be greatly enhanced by the addition of adenosine triphosphate to the fractionation medium. The synthetic capacity was not affected by the addition of ethylenediaminetetraacetic acid or by pre-incubation of the fraction with adenosine triphosphate. The presence of adenosine triphosphate in the fractionation medium resulted in a small increase in the ratio ribonucleic acid:protein of the supernatant fraction.

Storage of the fraction at $-15°$ resulted in a partial loss of activity. The activity could be restored by increasing the magnesium concentration in the incubation medium. A similar observation was reported by Gosselin & Popják (1959) in their study of reactivation of aged microsomes in a cholesterol-synthesizing system.

The supernatant fraction was centrifuged for 2 hr. at 105 000 *g* and this resulted in a loss of 36% of the ribonucleic acid and 60% of the synthetic activity. When the fraction was centrifuged after incubation of the [2-^{14}C]mevalonic acid a small pellet sedimented which contained 31% of the ribonucleic acid and 35% of the radioactive squalene.

When the incubation mixture was adjusted to pH 5·2, some 70% of the total ribonucleic acid was precipitated, along with a small amount of protein. Ninety-four per cent of the total radioactive squalene was recovered in this precipitate, whereas 70% of the total hydrophilic acid-labile radioactive compounds remained in the supernatant.

These data suggest that ribonucleic acid might be involved in the last stage of the squalene synthesis.

Bucher, N. L. R. & McGarrahan, K. (1956). *J. biol. Chem.* **222**, 1.

Gosselin, L. & Popják, G. (1959). *Biochem. J.* **71**, 660.

(3) A Comparison of the Reaction of Mono- and Di-functional Alkylating Agents with Nucleic Acids *in vitro* and *in vivo*

By P. Brookes and P. D. Lawley. (*Chester Beatty Research Institute, Fulham Road, London, S.W.* 3)

Following the preliminary experiments of Lawley (1957*a*, *b*) which indicated that $N_{(7)}$ of guanine moieties was the most reactive centre towards alkylation in nucleic acids, the reaction of deoxyribonucleic acid and ribonucleic acid with ^{35}S-labelled mustard gas (di-2-chloroethyl sulphide), was studied *in vitro* and *in vivo* (Brookes & Lawley, 1960). The principal reaction product was found to be 7-(β-hydroxyethylthioethyl)guanine, but another product, occurring to the extent of about 25% of the total reaction and having the properties of a cross-linked guanine, was also obtained both *in vitro* and *in vivo*. This product has been isolated but so far has not been purified sufficiently for chemical analysis. However, the corresponding product obtained with the nitrogen mustard, di-(2-chloroethyl)methylamine (HN 2), has been crystallized and by analysis shown to be di-(2-guanin-7-ylethyl)methylamine.

In view of the much greater tumour-growth inhibitory action of the two-armed as compared with the one-armed mustards (Loveless & Ross, 1950) it was of interest to study the reaction of ^{35}S-labelled 2-hydroxyethyl 2-chloroethyl sulphide (half mustard) with nucleic acids. The half mustard was found to react rapidly and extensively with nucleic acids and the reaction products were shown by chromatography and radioautography to be the same as obtained with mustard gas itself, except that the spot corresponding to the cross-linked material was absent.

Using the Ehrlich ascites tumour the full mustard was shown to be at least 30 times more effective than the half mustard as a tumour growth inhibitor.

As this evidence seemed to indicate that the cross-linked guanine product might be connected with the biological activity of the two-armed mustard a model of deoxyribonucleic acid was examined to see if this indicated the likely origin of the two guanines which are cross-linked. The model shows clearly that $N_{(7)}$ of guanine is sterically available for reaction and further indicates that cross-linking of two guanines on opposite twin strands by mustard gas could readily occur, but only if the sequence of bases is guanine–cytosine on one chain and in one direction only. Cross-linking in the opposite direction is sterically prohibited. This is in agreement with the experimental finding that only one-quarter of the alkylated guanines of deoxyribonucleic acid are cross-linked by mustard gas. However, it should be noted that a similar result is obtained for ribonucleic acid for which no model is at present available. Reaction of two adjacent guanines on the same chain seems less likely from the model but cannot be eliminated.

Brookes, P. & Lawley, P. D. (1960). *Biochem. J.* **77**, 478.

Lawley, P. D. (1957*a*). *Proc. chem. Soc.* p. 290.

Lawley, P. D. (1957*b*). *Biochem. biophys. Acta*, **26**, 450.

Loveless, A. & Ross, W. C. J. (1950). *Nature, Lond.*, **166**, 1113.

(4) The Difference Spectrum of Respiring Particles Isolated from the Hepatopancreas of *Carcinus maenas*

By R. B. Beechey. (*Department of Physiology and Biochemistry, The University, Southampton*)

The respiring particles used in these studies were prepared by homogenization of the hepatopancreas in 0·25M-sucrose. The homogenate was centrifuged at 850 *g* for 5 min. The supernatant fluid was then centrifuged for 10 min. at 8500 *g*. The pellet was then resuspended in fresh 0·25M-sucrose and recentrifuged at 8500 *g* for 10 min. The loosely packed upper layer of the final pellet was rejected. The more firmly packed light-buff-coloured material underneath was then suspended in a solution containing 5 mM-succinate, 5 mM-phosphate, pH 7·4, 5 mM-magnesium chloride, mM-adenosine triphosphate and sucrose to give a final tonicity of 0·25 osmolarity. The respiratory quotient of these suspensions was approximately 40 μl. of oxygen/hr./mg. of protein at 25°. The basic experimental procedure was that of Holton (1955). A Zeiss spectrophotometer PMQ II was used.

The difference spectrum (reduced minus oxidized) of these particles showed the following features.

(1) An absorption band with a peak at 345 mμ. This may be attributed to the reduced pyridine nucleotides.

(2) A deep trough over the range 380–433 mμ with minimum absorption at 410 mμ. This is the resultant of the absorbances of the $(a+a_3)'''\gamma$, $b'''\gamma$, $b'''\gamma$, $(c+c_1)''\gamma$ and $(c+c_1)'''\gamma$ bands.

(3) A strong absorption band from 433 to 455 mμ with a peak at 445 mμ which can be ascribed to the $(a+a_3)''\gamma$ band.

(4) A second broad trough stretches from 455 to 515 mμ. This is due to the absorption of the reduced flavoproteins and the absorptions of the oxidized cytochromes in this region.

(5) A minor plateau from 515 to 525 mμ corresponding to the $c''\beta$ and c_1'' bands.

(6) A small absorption band over the range 546–566 mμ containing the $b''\alpha$, $c''\alpha$ and $c_1''\alpha$ bands.

(7) An absorption band around 605 mμ due to the $(a+a_3)''\alpha$ band.

A detailed analysis of the 548–568 mμ region revealed the $c''\alpha$ band at 552 mμ, the $c_1''\alpha$ band at 554 mμ and the $b''\alpha$ band at 560–562 mμ. Other experiments localized the $(c+c_1)''\gamma$ band at 420 mμ and the $b''\gamma$ band at 430 mμ.

These results suggest that the particles contain a respiratory chain which has flavoproteins and cytochromes $a+a_3$, b, c and c_1 amongst its components.

The procedure for the isolation of the respiring particles was developed in collaboration with Dr K. A. Munday and Mr B. D. Thompson.

Holton, F. A. (1955). *Biochem. J.* **61**, 46.

(5) The Metabolism of Glyoxylate by Mammalian-Liver Mitochondria

By J. C. Crawhall and R. W. E. Watts. (*Medical Unit, St Bartholomew's Hospital, London, E.C.* 1)

Rat-liver mitochondria were incubated at 37° in Warburg flasks with sodium glyoxylate (6 mM) and L-glutamic acid (6 mM) at pH 7·4 and the rate of evolution of CO_2 was measured manometrically. No CO_2 was evolved in the absence of glutamic acid, indicating that the metabolism of glyoxylate could be proceeding by the glyoxalylglutamic acid pathway which Nakada & Sund (1958) suggested on the basis of their work with washed liver homogenates and a partially purified enzyme system. Using [1-^{14}C]glyoxylate, the radioactivity of the CO_2 recovered was less than that expected from the volume of CO_2 evolved. Using [1-^{14}C]-glutamic acid, it was found that about 20% of the CO_2 evolved was derived from the glutamic acid, though no CO_2 was evolved without glyoxylate present. A vigorous evolution of CO_2 occurred when mitochondria were incubated with glyoxylate and α-oxoglutarate. Mitochondria were incubated with [1-^{14}C]glyoxylate and L-glutamate in a series of flasks for increasing periods of time. The respired CO_2, glycine, oxalate and unchanged glyoxalate were isolated and counted. The results showed that glycine and oxalate were formed biosynthetically in this preparation. Attempts have been made to identify the degradative pathway of the C_2 component of glyoxylate. Experiments have been carried out with human-liver mitochondria and similar pathways have been found to be present.

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(6) The Need of the Isolated Perfused Goat Udder for Acetate and Glucose to Produce Milk

By D. C. Hardwick, J. L. Linzell and Susan M. Price. (*Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge*)

Acetate is generally accepted as the major source of energy for most ruminant tissues. Folley & French (1950) showed that ruminant mammary slices utilize acetate but not glucose and, since the R.Q. is over 1, deduced that acetate is used for fat synthesis. However, Linzell (1960) found that, in

conscious goats during lactation, the udder removes 3 times as much glucose as acetate from the blood.

We have now tested the effects of glucose and/or acetate on milk production by eight goat mammary glands, all of which received amino acids and minerals during perfusion by the method of Cargill-Thompson, Drury, Hardwick, Linzell & Tucker (1958), as modified by Hardwick & Linzell (1960). Two glands receiving glucose and acetate (3:1) produced milk for 12 hr. at 52 and 53% of the rate during the previous week. The average O_2 uptake during the experiments was 1·5 (0·5–2·6) ml. 100 g./min. and the R.Q. was 1·5 (0·8–2·8). Two glands receiving the equivalent weight of glucose only made 38 and 44% of milk for 13 and 10 hr. but the milk-fat concentration fell steadily after the second hour to 25 and 12% of normal. The O_2 uptake was 1·0 (0·6–1·4) ml./100 g./min. and the R.Q. was 0·8 (0·1–1·5). In two further experiments when acetate was given instead of glucose, milk secretion ceased, although the O_2 consumption was 0·85 (0·6–1·1) and the R.Q. 2·1 (1·3–2·9); after 3 and 4 hr., when glucose plus acetate was substituted for acetate, secretion started within an hour and thereafter was similar to the control. The first milk obtained after adding the glucose was high in casein and fat, suggesting that some synthesis had continued but that secretion had stopped during infusion of acetate.

Acetate plus glucose (9:1) or acetate plus fumarate (9:1) did not stimulate milk secretion and the O_2 uptake and R.Q. were similar to those in the presence of acetate only. There was a partial response to acetate plus glucose (2:1) and a further increase in O_2 uptake and milk secretion to the addition of glucose after 5 hr.

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(7) Triphosphopyridine-linked Dehydrogenases in the Adrenal Cortex in Man-Effect of Corticotrophin and Distribution of Enzymes

By G. P. Studzinski, T. Symington and J. K. Grant. (*University Departments of Pathology and Steroid Biochemistry, Royal Infirmary, Glasgow, C.* 4)

The unusually high glucose 6-phosphate dehydrogenase activity found in the adrenal cortex of the ox and rat (Kelly, Nielson, Johnson & Vestling, 1955) and the fact that this is linked with the production of reduced triphosphopyridine nucleotide (TPNH) required for adrenocortical steroid biosynthesis (Grant & Brownie, 1955) prompted an investigation of the effect of corticotropin preparations administered *in vivo* on the TPN-linked dehydrogenations in the human adrenal cortex *in vitro*. Adrenal glands were obtained from breast-cancer patients undergoing two-stage bilateral adrenalectomy. The gland removed at the first operation, without any hormone administration, served as a control. Corticotropin preparations were administered intramuscularly before the second operation. TPN-reducing dehydrogenases were assayed by the methods of Glock & McLean (1953) (glucose 6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase) and Ochoa (1955) (isocitric dehydrogenase and malic enzyme) in the supernatant fraction obtained by centrifuging homogenates of glands at 18 000 *g* for 30 min.

Administration of Armour adrenocorticotrophic hormone (ACTH) gel produced a twofold increase in glucose 6-phosphate dehydrogenase, a less-marked increase in 6-phosphogluconic acid dehydrogenase and no increase in activity of isocitric dehydrogenase. A rather less pure preparation of Organon ACTH gave similar results, including a marked increase in activity of malic enzyme. Both preparations produced an approximately twofold increase in weight of the human adrenal glands. In an attempt to find if the enzyme changes were related to steroid biogenesis or adrenal gland growth, ACTH A_1+A_2 (Dixon & Stack-Dunne, 1955) almost free from adrenal gland growth-promoting activity was used. Similar increases in the activity of glucose 6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase were observed but malic enzyme activity did not increase. Gland weights did not increase significantly.

Increases in enzyme activity were paralleled by the usual histological changes seen in the human adrenal cortex after stress or administration of corticotropin (Symington, Duguid & Davidson, 1956).

Separation of the homogenates used into nuclear mitochondrial, microsomal and supernatant fractions by the procedure of Schneider (1948), shows that the human adrenal does not differ from other tissues previously studied, in that the TPN-linked dehydrogenases are located almost exclusively in the supernatant fraction.

Distribution of glucose 6-phosphate dehydrogenase throughout the adrenal cortex was measured by determining this enzyme activity in 100 μ sections cut parallel to the capsule. In glands removed without corticotropin administration, the enzyme appears to be uniformly distributed

throughout the cortex. After corticotropin administration the enzyme shows a distinct maximum activity in a region corresponding to the junction of the fascicular and reticular zones as determined by histological examination of the whole adrenal cortex.

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(8) Glycine Metabolism in Acute Porphyria

By F. F. Richards and J. J. Scott. [*Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2, and the Department of Clinical Medicine, Malmö General Hospital (University of Lund, Sweden)*]

In order to obtain information on the nature of the metabolic disorder of acute porphyria, glycine metabolism was studied in five healthy and nine porphyric subjects. The average output of δ-aminolaevulic acid (mg.) + porphobilinogen (mg. equivalent to δ-aminolaevulic acid) by the healthy subjects was $4{\cdot}06 \pm 1{\cdot}77$ mg./day; 7·6 mg./day being taken as an arbitrary upper limit of normal.

Following the oral administration of two successive doses of 25 g. of glycine at 4 hr. intervals, there were no adverse clinical effects, while the rate of urinary excretion of δ-aminolaevulic acid and porphobilinogen increased substantially in seven out of nine patients, but remained within normal limits in all the healthy subjects. This increase was demonstrated in a porphyric patient with extensive peripheral neuropathy, who at the time of the test had a rate of urinary excretion of δ-aminolaevulic acid and porphobilinogen which was close to normal.

Glycine to serine conversion was studied in six patients with acute porphyria by estimating plasma glycine and serine concentrations after a 25 g. dose of oral glycine (Butterworth, Santini & Perez-Santiago, 1958). This conversion was normal in three patients and markedly reduced in three other patients.

Butterworth, C. E., Santini, R. & Perez-Santiago, E. (1958). *J. clin. Invest.* **37**, 20.

(9) A Micromethod for Concentrating Proteins in Cerebrospinal Fluid

By J. Colover* (introduced by P. N. Campbell). (*Department of Neurology and Institute of Clinical Research, The Middlesex Hospital, London, W. 1*)

Although a number of methods have been described previously for concentrating cerebrospinal fluid so that the protein components may be examined by electrophoresis, there are difficulties in their routine use. Thus double dialysis followed by freeze-drying is cumbersome, time-consuming, and it may be difficult to recover the small amount of dried protein. The method of Mies (1952) requires at least 5 ml. of fluid, as does the ultrafiltration method of Esser & Heinzler (1952). In both of these procedures material may be lost on the membranes by drying.

For an immunological investigation of the proteins of the cerebrospinal fluid in human demyelinating disease, a method of concentrating 0·5 ml. samples of fluid was required. A special chamber was constructed of plastic and glass so that it had two compartments separated by a membrane. In one compartment, namely the lower one, is placed 0·5 ml. of cerebrospinal fluid and into the other or upper compartment is placed 1 ml. of 30 % (w/w) polyethyleneglycol solution (Carbowax 20 M., G. Gurr) in distilled water. Cellulose membrane separates the two compartments allowing water, salts and electrolytes to diffuse out of the cerebrospinal fluid into the polyethylene glycol. The apparatus is kept at 4° overnight for 16 hr. in a moist chamber to prevent evaporation. After this time the cerebrospinal fluid has usually been concentrated to a volume of between 5 and 20 μl. Most of the concentrate accumulates in a ring-shaped groove placed in the floor of the compartment which holds the cerebrospinal fluid, from which it can be withdrawn by means of a capillary tube. It is then mixed with 5 μl. of barbitone buffer pH 8·6, *I* 0·05, and applied to cellulose acetate membrane for electrophoresis by the method of Kohn (1957), in the same buffer for $2\frac{3}{4}$ hr. at 0·5 mA/ml. of strip width. After drying in an oven for 30 min. at 100° the strips are stained with naphthalene black in 10 % (v/v) acetic acid in methanol, and then examined in a scanning instrument (Evans Electroselenium Ltd.) connected to an external Unigalvo galvanometer.

By this means it is possible to recognize and measure pre-albumin, albumin, α_1-, α_2-, β- and γ-globulins. The β band is often duplicated. Cerebrospinal fluid containing as little as 15 mg./100 ml. of protein has been examined successfully.

* Member of the External Scientific Staff, Medical Research Council.

The apparatus is easy to clean and unlike the ultrafiltration methods mentioned above, the membrane in it is used only once and then discarded.

It is a great pleasure to thank the clinical research committee of The Middlesex Hospital and Dr Michael Kremer for facilities for doing this work.

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(10) Demonstration of the Non-identity of the Pancreatic Enzymes Hydrolysing Triolein and Tween 20, and the Purification of 'Tweenase'

By R. A. Evans and D. A. Stansfield. (*Department of Agricultural Chemistry, University College of North Wales, Bangor, Caerns.*)

The assay method for lipase which is based upon the use of Tween 20 (polyoxethylene sorbitan monolaurate) was introduced by Archibald (1946) and is recommended in *Methods in Enzymology* by Bier (1955). It has been used in the estimation of so-called pancreatic lipase by, among others, Boissonas (1948). Its use as a substrate for lipase has been criticized by Desnuelle (1951) on the grounds that the only substrate with which one can estimate lipase is a higher triglyceride.

During investigations of the behaviour of the esterolytic activities present in pig-pancreas homogenates using a pH-stat (Radiometer, Ltd., Copenhagen, Denmark) it was found that the hydrolyses of triolein and of Tween 20 were sensitive to different inhibitors. The enzyme hydrolysing triolein (lipase) was inhibited by 0·2 mM-*p*-chloromercuribenzoate, but was unaffected by the same concentration of *NN'*-di-*iso*propyl phosphodiamidic fluoride (mipafox), whereas that which hyrolysed Tween 20 (Tweenase) was not inhibited by 0·2 mM-*p*-chloromercuribenzoate, but was completely inhibited by the same concentration of mipafox. In addition, Tweenase was much more sensitive to heat than lipase.

By lowering the pH of these homogenates from 6·3 to 5·3, it was possible to precipitate all the lipase activity away from the Tweenase, showing conclusively that the activities towards these substrates are due to different enzymes. This separation was also achieved during a purification of Tweenase, starting from pancreatin, in which the activities towards ethyl acetate, triacetin, acetyl choline and benzoyl choline all decreased relative to that towards Tween 20.

The purification, which consisted of salt, pH and dielectric manipulation of proteins extracted from pancreatin by means of an ultrasonic disintegrator, (M.S.E./Mullard), yielded a product between 3 and 4·5 times more pure than that obtained by Boissonas (1948) and in 4 times greater yield per unit of starting material.

It is thus concluded that Tween 20 is not a substrate for pancreatic lipase, and we suggest that it should no longer be used as such.

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(11) Diauxie in the Induction of Arylsulphatase Synthesis in Fungi

By T. Harada and B. Spencer. (*Institute of Scientific and Industrial Research, Osaka University, Japan, and the Department of Biochemistry, University of Wales, Cardiff*)

Of twenty-seven fungi tested only two strains (both *Saccharomyces cerevisiae*) failed to release *p*-nitrophenol from *p*-nitrophenyl sulphate when this arylsulphate was used as the sole source of sulphur in a chemically defined medium. When potassium sulphate was included in the medium as an additional sulphur source the release of *p*-nitrophenol was markedly diminished, and in many cases completely repressed, in all but four strains, *Rhizopus stolonifer*, *Pichia membranefaciens*, *Aspergillus nidulans* and *Torulopsis utilis*. Cysteine had a similar effect to potassium sulphate but choline sulphate and taurine were not inhibitory towards *p*-nitrophenol liberation.

The arylsulphatase activity *in vitro* of acetone-dried powders of the mycelium of four selected fungi grown on different media showed a similar pattern to the results *in vivo*. Maximum arylsulphatase activity was obtained when taurine was the sole sulphur source and comparable activities were produced by choline sulphate and *p*-nitrophenyl sulphate. Cysteine, methionine, potassium sulphate and potassium sulphite diminished arylsulphatase activity to 10% or less of that of the same strains grown on taurine. Further experiments showed that twenty-three of twenty-seven tested strains produced arylsulphatase activity when grown on a chemically defined medium containing taurine as the sole sulphur source while a large number of these strains were unable to produce the enzyme on a glucose–peptone–yeast extract medium.

From currently accepted schemes of the utilization of SO_4^{2-} for growth purposes it can be seen that the sulphur sources which are ineffective for arylsulphatase production are those which are concerned in the metabolism of SO_4^{2-} to methio-

nine. Taurine, choline sulphate and arylsulphates are not direct intermediates in this metabolic pathway.

These results were discussed in terms of a diauxie phenomenon (Monod, 1941; Cohn & Horibata, 1959) concerning the utilization of sulphur compounds.

Cohn, M. & Horibata, K. (1959). *J. Bact.* **78**, 624.
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(12) Reduced uptake of [^{14}C]streptomycin by Bacteria in the Presence of Respiratory Inhibitors and under Anaerobic Conditions

By R. Hancock. (*Department of Bacteriology, University of Manchester, Manchester* 13)

The uptake of [^{14}C]streptomycin by cells of a number of bacterial species has been investigated. In aerobically growing cultures, uptake of radioactivity by all the organisms used follows closely the increase in cell mass. When growth is fully inhibited, the radioactivity taken up, using streptomycin at minimal bactericidal concentrations (in brackets), corresponds to 0·06 μg. of streptomycin/mg. of cells for *Bacillus megaterium* KM (1 μg./ml.); 1·5 μg. of streptomycin/mg. of cells for *Staphylococcus aureus* Duncan (10 μg./ml.); and 1·6 μg. of streptomycin/mg. of cells for *Bacillus subtilis* (35 μg./ml.). The uptake by *B. megaterium* corresponds to about 5×10^4 molecules of streptomycin/cell; cells of a streptomycin-resistant strain took up less than 1% of this quantity. The radioactivity is not displaced by unlabelled streptomycin; it can be extracted by cold trichloroacetic acid (5%, w/v) but not by hot water. When protoplasts of *B. megaterium* are fractionated by the procedure of Weibull, Beckman & Bergström (1959), the cytoplasmic fraction contains over 95% of the radioactivity, which can be sedimented at 105 000 *g* for 4 hr.

The rate of uptake by *Staphylococcus aureus* growing anaerobically, or in an atmosphere of 80% carbon monoxide/20% air [when streptomycin at this concentration is not bactericidal (Lightbown, 1957; Hancock, 1960)] is only 12%, or 13·5% respectively, of that under aerobic conditions. In the presence of 2-heptyl 4-hydroxyquinoline *N*-oxide (0·4 μg./ml.), the growth inhibitory effect of streptomycin (25 μg./ml.) on *Bacillus subtilis* is completely antagonized and the uptake of radioactivity is only about 25% of that in the absence of the *N*-oxide.

These results suggest that increased resistance of these organisms to streptomycin during anaerobic growth, or in the presence of carbon monoxide or 2-heptyl 4-hydroxyquinoline *N*-oxide [an inhibitor of respiration via the cytochrome system (Lightbown & Jackson, 1956)] does not indicate that the streptomycin-inhibited process is one on which the organisms are more dependent during aerobic respiration; but that uptake of streptomycin into the cell is associated with aerobic respiratory processes and is reduced when these are depressed.

[^{14}C]Streptomycin was a generous gift from Dr H. B. Woodruff, of Merck, Sharpe and Dohme Research Laboratories, Rahway, New Jersey, U.S.A.

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(13) The Effects of Amphotericin and Nystatin on Intermediary Metabolism in Baker's Yeast

By R. A. Steggle, F. L. Jackson, V. Moses* and M. J. H. Smith. (*Departments of Chemical Pathology and Bacteriology, King's College Hospital Medical School, London, S.E.* 5)

Amphotericin-B (Fungizone) and nystatin (Mycostatin) are antibiotic substances which show activity against a range of fungal organisms and are used in the treatment of yeast infections in man.

Baker's yeast was incubated aerobically for 30 min. at 25° in 2 mM-phosphate buffer, pH 7·8, with $NaH^{14}CO_3$, [2-^{14}C]acetate, or [^{14}C]glucose, in the presence or absence of sodium deoxycholate (0·6 mg./ml.), amphotericin (0·75 mg./ml.) plus deoxycholate, or nystatin (0·3 mg./ml.) plus deoxycholate. The deoxycholate was included in order to solubilize the antibiotics. The cells were killed, extracted with ethanol, the extracts chromatographed, and radioactive substances located by radioautography and counted with an end-window Geiger-Müller tube (Moses & Smith, 1960).

The presence of deoxycholate, alone or together with amphotericin, had no effect on the total incorporation of ^{14}C or on the subsequent distribution of radioactivity among the soluble intermediates of the yeast metabolizing either the labelled bicarbonate or acetate. However, nystatin plus deoxycholate caused 80% and 50% inhibition respectively, of the total incorporations of ^{14}C from $NaH^{14}CO_3$ and [2-^{14}C]acetate; the percentage distribution of ^{14}C among the soluble intermediates was not affected. The results with [^{14}C]glucose were more complex. Deoxycholate alone caused a

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40% inhibition of total incorporation of ^{14}C, due mainly to a fall in radioactivity in the phosphorylated compounds. With amphotericin plus deoxycholate, the total uptake of ^{14}C was not altered, but 40% more radioactivity was found in the amino acids and less in sugar phosphates, uridine diphosphate glucose, and trehalose. Nystatin plus deoxycholate caused a 60% inhibition of the total incorporation of ^{14}C; radiocarbon almost disappeared from the sugar phosphates and uridine diphosphate glucose and was reduced to about 25% of the control value in trehalose, but more ^{14}C was found in the amino acids, particularly glutamate.

It is possible that amphotericin interferes with protein synthesis, thus causing a generalized accumulation of labelled amino acids. Nystatin also produced some accumulation of radiocarbon in amino acids, but its main action appeared to be concerned with energy-yielding processes as evidenced by the very low incorporation of ^{14}C into sugar phosphates, uridine diphosphate glucose and trehalose.

We are grateful to the Smith, Kline and French Research Institute for a grant, and to E. R. Squibb and Sons Ltd., for gifts of amphotericin-B and nystatin.

Moses, V. & Smith, M. J. H. (1960). *Biochem. J.* **76**, 585.

(14) Carboxylation of Propionyl-Coenzyme A by Extracts of *Rhodospirillum rubrum*

By Jane Gibson and M. Knight. (*Agricultural Research Council Unit for Microbiology, and Department of Microbiology, The University, Sheffield* 10)

Short-exposure experiments, based on the methods used by Calvin (Bassham & Calvin, 1957), have been used to study the photometabolism of propionate by *Rhodospirillum rubrum*. Kinetic studies of the intermediates have shown that [2-^{14}C]propionate is converted first into succinate. Similarly, succinate is the first labelled product formed from $^{14}CO_2$ and unlabelled propionate. Degradation of the intermediates has shown that [2-^{14}C]propionate is converted into [2:3-$^{14}C_2$]-succinate, fumarate and malate; and that $^{14}CO_2$ goes into the carboxyl groups of these compounds. These results suggest that the first step in propionate metabolism might be a carboxylation similar to that found in animal tissues by Flavin & Ochoa (1957).

Cell-free extracts of *R. rubrum* have been prepared which fix $^{14}CO_2$ in the presence of propionyl coenzyme A and adenosine triphosphate under the conditions used by Flavin & Ochoa (1957). The fixation of $^{14}CO_2$ was measured using a gas-flow proportional counter with propane as the counting gas. The extracts were prepared by ultrasonic disintegration, followed by removal of chromatophores in a Spinco preparative ultracentrifuge, and ammonium sulphate fractionation. Adenosine triphosphate, Mg^{2+} and propionyl coenzyme A were essential components of the system. The preparation of glutathione available inhibited the reaction and in later experiments it was omitted from the reaction mixture.

The products of the carboxylation of propionyl coenzyme A were studied in order to try to identify methylmalonate, since Flavin, Ortiz & Ochoa (1955) showed that methylmalonyl coenzyme A is the first product of the carboxylation reaction in animal tissue. When an ether extract of the reaction mixture was chromatographed on paper in a solvent which separates succinate from methylmalonate, three radioactive spots were found. The largest of these was succinate. Of the other two, one has been identified as methylmalonate by co-chromatography on paper in two dimensions with inactive methylma onate.

It was reported by Swick & Wood (1960) that propionyl coenzyme A carboxylases in other organisms are sensitive to inhibition by avidin and that this inhibition is reversed by biotin, suggesting that biotin may be a cofactor for this enzyme. Fixation of $^{14}CO_2$ was completely inhibited in these *Rhodospirillum* extracts by pre-incubating the enzyme with avidin. This inhibition could be prevented by first treating the avidin with an excess of biotin.

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Swick, R. W. & Wood, H. G. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 28.

(15) Deamination of Amino Acids in *Rhodopseudomonas spheroides*

By D. S. Hoare. (*Agricultural Research Council Unit for Microbiology, The University, Sheffield* 10)

The deamination of amino acids by *Rhodopseudomonas spheroides* has been studied as part of an investigation on the amino acid metabolism of photosynthetic bacteria. *R. spheroides* was grown semi-anaerobically in the light and crude cell-free extracts were prepared by sonic disintegration and centrifugation at 19 000 *g*. Such extracts formed pyruvate on incubation with D- or L-alanine at 30° with an optimum pH 7·8. After centrifuging crude extracts for 2 hr. at 105 000 *g*, deaminating activity

was recovered in ammonium sulphate fractions precipitated between 30 and 45% saturation. Alanine deamination then showed an obligatory requirement for ferricyanide as electron acceptor; pyridine nucleotides were inactive. Ferricyanide reduction has been followed spectrophotometrically at 420 mμ and corresponds to the reduction of 2 moles of ferricyanide per mole of pyruvate formed. L-Glutamate is not deaminated, although a number of L-amino acids including leucine, which is the most active, are deaminated even in the absence of ferricyanide. Alanine deamination is inhibited competitively by very low concentrations of semicarbazide and hydroxylamine. Deamination of leucine is also inhibited.

Further purification has been achieved by adsorption and elution from calcium phosphate gel, resulting in a 10- to 15-fold purification for alanine and a 20- to 30-fold purification for leucine deamination. With a mixture of alanine and leucine there is a partial summation of activities. The results of these investigations suggest that two enzymes are involved; a specific enzyme for the deamination of L-alanine, which does not use oxygen as terminal acceptor, and a non-specific deaminating system for L-leucine and a number of L-amino acids, which uses oxygen as terminal electron acceptor.

The extreme sensitivity of both reactions to hydroxylamine contrasts sharply with the amino acid oxidase reactions. Burton (1951) found that L-amino acid oxidase from *Neurospora crassa* (followed manometrically by oxygen uptake) was stimulated by 10 mM-hydroxylamine owing to inhibition of catalase. The sensitivity of these deaminating systems to carbonyl reagents, and the reports of non-enzymic deamination of amino acids by pyridoxal and metal ions (Ikawa & Snell, 1954) suggest the possibility that the deaminating systems of *R. spheroides* are pyridoxal dependent.

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Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 4900.

(16) Nitric Oxide Reductase from *Pseudomonas aeruginosa*

By C. A. Fewson and D. J. D. Nicholas.* (*Long Ashton Research Station, University of Bristol*)

Several workers have shown that nitric oxide is produced during denitrification (Najjar & Allen, 1954; Chung & Najjar, 1956*a*; Walker & Nicholas, 1960) and its reduction to nitrogen has been demonstrated in several bacteria (Baalsrud & Baalsrud, 1954; Chung & Najjar, 1956*b*; Iwasaki, Matsubayashi & Mori, 1956). Chung & Najjar (1956*b*) suggested that nitric oxide reductase from *Pseudomonas stutzeri* requires iron and copper for its activity, but in this communication we show that the enzyme from *P. aeruginosa* is a flavoprotein containing iron only.

Actively denitrifying cells of *P. aeruginosa* (NCIB 8704) grown in a nitrate–peptone medium (Verhoeven, 1952) under anaerobic conditions were harvested and disrupted with an ultrasonic probe (20 kcyc./sec.). More than 90% of the enzyme was in the supernatant solution prepared by centrifuging the homogenate at 20 000 *g* for 30 min. The enzyme was purified 25-fold by ammonium sulphate precipitation and by separation on a column of diethylaminoethylcellulose. The purified enzyme, which did not utilize reduced pyridine nucleotides, was assayed with reduced pyocyanine as the hydrogen donor. Maximum activity was at pH 8·0. The activity, which was reduced by repeated precipitation of the enzyme with ammonium sulphate (pH 7·0), was restored by either flavin mononucleotide or flavinadenine dinucleotide ($K_m = 4 \times 10^{-5}$ M). Compounds which are known to chelate iron and copper, viz. α,α′-dipyridyl, *o*-phenanthroline, potassium ethyl xanthate and 2:9-dimethyl-1:10-phenanthroline, all reduced the enzyme activity by more than 40% at 2×10^{-3} M. Only a slight inhibition (< 10%), however, was produced by sodium diethyldithiocarbamate, salicylaldoxime or α-benzoin oxime which chelate copper but not iron between pH 7 and 8 (Hallaway, 1959). Enzyme activity was diminished by 75% in cells deficient in iron, whereas it was unchanged in those deficient in copper, molybdenum, manganese or zinc.

Nitric oxide, or a compound with which it equilibrates, is likely to be an intermediate in assimilatory nitrate reduction as well as in the dissimilatory process since we find that it is utilized by bacteria, fungi, algae and higher plants which also have an active nitrate reductase. Microorganisms grown on ammonium nitrogen, however, show little or no nitric oxide reductase activity. These results are in agreement with those of McNall & Atkinson (1956) who demonstrated that *Escherichia coli* (Bn) which was adapted to grow on nitrate could also utilize nitric oxide as the sole nitrogen source for growth.

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* Member of the Scientific Staff of the Agricultural Research Council seconded to the University of Bristol.

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(17) Hydroxylamine Reductase from *Pseudomonas aeruginosa*.

By G. C. Walker and D. J. D. Nicholas.* (*Long Ashton Research Station, University of Bristol*)

Hydroxylamine has been found to be an intermediate in the reduction of nitrate to ammonia by bacteria and fungi (Woods, 1938; Back, Lascelles & Still, 1946; Verhoeven, 1952; Egami, Yamada & Taniguchi, 1952; Nason, Abraham & Averbach, 1954). Taniguchi *et al.* (1958) also found hydroxylamine reductase in several denitrifying bacteria.

In the present work a 47-fold purification of the enzyme from *Pseudomonas aeruginosa* was obtained by ammonium sulphate precipitation and treatment with calcium phosphate gel. The purified enzyme specifically reduced hydroxylamine to ammonia (K_m, $NH_2OH = 2{\cdot}9 \times 10^{-4}$ M) when either reduced pyocyanine (PyH_2), reduced methylene blue (MbH_2) or reduced flavin [riboflavin-H_2, flavin mononucleotide-H_2 ($FMNH_2$), flavinadenine dinucleotide-H_2 ($FADH_2$)] was the donor (K_m, $PyH_2 = 1{\cdot}2 \times 10^{-8}$ M; K_m, $MbH_2 = 4{\cdot}4 \times 10^{-8}$ M; K_m, $FADH_2 = 10^{-8}$ M). Neither reduced di- or tri-phosphopyridine nucleotide nor reduced cytochrome *c* were effective donors. Hydroxylamine reductase activity, determined by following the oxidation of MbH_2, was inhibited competitively by hydrazine, although the latter did not oxidize MbH_2.

The purified enzyme contained 0·9 μm-mole of FAD/mg. of protein. The native flavin was partly removed by precipitating the enzyme, three times, with ammonium sulphate at pH 6·0. When either PyH_2 or MbH_2 was the donor, the treated enzyme was reactivated by returning FAD (25 μmM), but FMN was without effect.

Metal-deficiency studies showed that the enzyme activity was markedly reduced in extracts of felts deficient in manganese. Molybdenum and copper deficiencies were without effect, but a deficiency of iron reduced activity by 30%. Although purified enzyme fractions contained a cytochrome of the *c* type, there is no evidence that it functioned in electron transfer during enzyme action. The dialysed enzyme was activated three-fold by manganese (0·2 μM) after dialysing it first against 1 mM-sodium diethyldithiocarbamate in 10 mM-phosphate (pH 7) and then against phosphate alone for a further 18 hr. to remove the chelating agent. Although cobalt (0·5 μM) substituted for manganese it was only one-tenth as effective. At similar concentrations (0·5 μM), copper and zinc inhibited the dialysed enzyme, whereas iron, magnesium and nickel were without effect. The activation of the dialysed enzyme by manganese was inhibited competitively by *p*-chloromercuribenzoate, suggesting that –SH groups may be involved in binding the metal to the enzyme.

* Member of the Scientific Staff of the Agricultural Research Council, seconded to the University of Bristol.

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(18) Comparison of the Effect of Vitamin B_{12} on the Metabolism of ^{14}C-Labelled Formate and Propionate by *Ochromonas malhamensis*

By A. M. White and H. R. V. Arnstein. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Ochromonas malhamensis has a specific requirement for vitamin B_{12} as a growth factor, and it therefore provides a convenient system for making comparisons between various metabolic pathways which, in other organisms, and in the mammal, are thought to be influenced by vitamin B_{12}, e.g. biosynthesis of methyl groups (Arnstein, 1958) and deoxyribosides (Downing & Schweigert, 1956), isomerization of methylmalonate to succinate (Stadtman, Overath, Eggerer & Lynen, 1960).

When cells were grown with limiting amounts of cyanocobalamin (0·03 μmg./ml.), harvested by centrifuging, washed and incubated in water at 30° for 1 hr. with sodium [^{14}C]formate, there was greater incorporation of the label into protein methionine and serine and less oxidation to carbon dioxide in the presence of cyanocobalamin than in its absence; but the differences are small and only of the same order as those previously reported for the incorporation of amino acids into protein (Arnstein & White, 1959). In oxidation experiments with labelled propionate, however, cyanocobalamin increased the radioactivity of carbon dioxide 50- to 100-fold after a 1 hr. incubation with sodium [2-^{14}C]propionate and increased it

10-fold when sodium [1-^{14}C]propionate was used. The oxidation of sodium [2-^{14}C]acetate was affected only by a factor of about 2, i.e. to about the same extent as protein biosynthesis. These results suggest that the major role of vitamin B_{12} in *O. malhamensis* may be in the propionate-to-succinate pathway, presumably as a cofactor for methylmalonyl coenzyme A isomerase (Stadtman *et al.* 1960).

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(19) Studies on the Mode of Action of Vitamin A. 1. The Metabolism, Composition and Degradation of Chick-limb Cartilage *in vitro*

By Honor B. Fell, J. A. Lucy and J. T. Dingle. (*Strangeways Research Laboratory, Cambridge*)

Fell & Mellanby (1952) showed that in organ cultures the addition to the medium of excess vitamin A severely affects cartilaginous limb-bone rudiments from 6- to 7-day chick embryos. During 7–10 days' cultivation, the vitamin-treated rudiments cease to grow, begin to shrink and become soft and gelatinous; the matrix no longer stains metachromatically with toluidine blue and the intercellular partitions become narrow and eventually disappear. The object of the present investigation was to find what biochemical changes are associated with these morphological effects.

After 6 days' cultivation in medium containing added vitamin A, the wet and dry weights and amino sugar content of the limb-bone rudiments were half those of the controls whilst the ribonucleic acid phosphorus had fallen to 40% of the control value; the deoxyribonucleic acid phosphorus, however, was 80% of the control level. The vitamin-treated explants also showed a lowered rate of oxygen uptake.

Experiments were made to find whether normal cartilage contains an enzyme capable of producing the 'vitamin A effect' on the matrix. It was found that after treating the rudiments with water at 4° for 1 hr. followed by incubation in buffer for 2 hr. at 37° at pH 3–5, the metachromatic staining was greatly reduced, half the hexosamine was lost and protein components were liberated into the buffer. Above and below pH 3–5 there was little effect on the cartilage matrix.

It is thought that the effects of excess vitamin A could be explained on the hypothesis that the vitamin alters the permeability of the intracellular particles, e.g. the mitochondria and lysosomes.

Fell, H. B. & Mellanby, E. (1952). *J. Physiol.* **116**, 320.

(20) Studies on the Mode of Action of Vitamin A. 2. The Release of a Bound Protease by the Action of Vitamin A

By J. T. Dingle and J. A. Lucy. (*Strangeways Research Laboratory, Cambridge*)

Treatment of bone rudiments with water followed by incubation in buffer caused marked changes in the extracellular material. The pH dependence of these effects suggested that an enzyme had been released by the hypotonic treatment. The water extract was found to contain a protease with an acid pH optimum. A protease with a similar pH optimum is located in the cytoplasmic particles obtained by centrifugal fractionation of embryonic cartilage, and can be liberated by hypotonic treatment.

These findings prompted the investigation of the action of vitamin A upon subcellular particles. Rat liver was chosen as a convenient source of material as Gianetto & de Duve (1955) have shown that stable cathepsin-containing particles, the lysosomes, can easily be obtained from this tissue.

Rat-liver lysosomes suspended in 0·25M-sucrose and treated with vitamin release a proteolytic enzyme with an acid pH optimum. This action of the vitamin is dependent upon both temperature and pH, and was not found below 30° or below pH 5·0. The sensitivity of the reaction appears to depend partly upon the amount of non-lysosomal material present. In preparations largely freed of mitochondria, 0·25 μg. of vitamin A/mg. of original tissue, released 50% of the activity liberated by hypotonic treatment. The vitamin had no action on the activity of the enzyme freed by hypotonic treatment. The pH optimum of the enzyme released by both hypotonic and vitamin treatment is the same.

The above results may indicate the mechanism by which the vitamin, acting at a subcellular level, is able to alter so drastically the composition of the extracellular material.

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[*The Editors of* The Biochemical Journal *accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **400th Meeting** *of the Society was held in The Middlesex Hospital Medical School, London, W. 1, on Friday,* 18 *November* 1960, *when the following papers were presented:*

COLLOQUIUM ON HISTOCHEMISTRY

Rate of Interaction of Cytochemical Substrates with Enzymes Present in Fixed Tissue.

By D. G. O'Sullivan (introduced by S. J. Holt). (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W. 1*)

Unless tissue is separated into minute parts by either homogenization-centrifugation processes or by microdissection, the only promising approach to information on the precise topology of enzymic activity and kinetics is microphotometric study of stain-density development in cytochemical staining methods. Unfortunately, the stain-production process is usually indirect and, consequently, subject to a variety of complicating features. Naturally, accurate localization is a prime necessity in kinetic studies (Holt & O'Sullivan, 1958; O'Sullivan, 1956) but, in addition, the immediate product of the enzymic reaction should be converted very rapidly into the dye alone and no side reactions should occur. The rate of formation of immobilized stain should be very close to the rate of substrate removal or be simply related to it, i.e. not only must the enzymic reaction be the rate-controlling stage, but stain production should possess simple and suitable kinetics.

Penetration of reagents into tissue slices of the thickness normally used in cytochemistry is usually very rapid. For example, it takes only 0·25 sec. for the concentration at the centre of a slice 10 μ thick to reach 90% of the (constant) external concentration if the diffusion coefficient is 10^{-6} cm.^{2} sec.$^{-1}$. Diffusion coefficients will be greater than 10^{-6} if adsorption of substrate on to protein does not occur and if no special barriers of low permeability are present. The corresponding time for a tissue slice 1 mm. thick is 42 min., so that penetration is a more serious problem with thick pellets used for electron microscopy.

If a substrate penetrates rapidly, its average rate of removal in a region of tissue is independent of whether the distribution of enzyme is uniform or irregular. Consequently, cytophotometric measurements can give the rate of removal of substrate that would occur if the enzyme were uniformly distributed in that region. Thus, in principle, initial rates of removal of substrate in a given (small) region of tissue are determinable with a range of increasing substrate concentrations and an effective Michaelis–Menten constant obtained.

If slow diffusion of substrate occurs in a non-uniform enzyme distribution, then there is little hope of disentangling information on the enzyme kinetics *in situ*. However, uniform enzyme distribution, in regions of simple shape, bounded by a membrane of low permeability can be analysed if a steady state is rapidly attained (Blum & Jenden, 1957). Then measurements of rates of increase of stain density can give information on both the Michaelis–Menten constant and the membrane permeability.

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O'Sullivan, D. G. (1956). *Bull. math. Biophys.* **18**, 199.

Possible Quantitative Methods for Studies of Enzymes in Single Cells.

By A. J. Hale. (*Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C. 2*)

There are at present available three methods which permit accurate measurement, at the microscopic level, of the amounts of material in single cells. They are absorption methods at visible and ultraviolet wavelengths, absorption methods at X-ray wavelengths, and measurements of optical path differences at visible wavelengths. The limitations of these methods have been thoroughly investigated and their accuracy determined (Davies, 1958; Engström, 1958; Hale, 1958; Walker & Richards, 1959). Use of these methods for enzyme studies would depend upon visualization of the enzyme by means of a reaction product. The only attempts that have been made to apply these techniques in the study of enzymes are those of Barter, Danielli & Davies (1955) using microinterferometry, and Thorell & Chance (1960) using microspectrophotometry.

In making measurements at the cellular level certain physical characteristics such as size, form, extinction coefficient and refractive index of the absorbing or retarding reaction product must be considered. Using the information which we have

as the result of investigations of other types of cytochemical reactions we can define some ideal physical characteristics. If the reaction product absorbs light then it must be amorphous and of low extinction (less than 0·1). The degree of homogeneity of its distribution in the object is unimportant, within limits. It should have a specific spectral absorption curve and it should not change its extinction coefficient at high concentrations. It should absorb at wavelengths other than those absorbed by the object itself. At X-ray wavelengths the product must have a high extinction coefficient and a characteristic absorption edge in the long wavelength region. For interferometry the product must have a measurable specific refractive increment, and should not be birefringent or absorbing.

Satisfaction of these criteria will permit accurate measurement of the product of a reaction with an enzyme if accurate instruments are used. For quantitative measurement of enzyme kinetics, or total amount of enzyme present, then additional criteria of specificity and stoicheiometry between enzyme and reaction product and of stability and substantivity of the latter must be satisfied.

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Properties of Tetrazolium Salts and Alternative Electron Acceptors as Factors in Dehydrogenase Histochemistry.

By A. G. E. Pearse. (*Postgraduate Medical School, London, W.* 12)

Most histochemical dehydrogenase reactions employ a ditetrazolium salt [2:2′-di-*p*-nitrophenyl-5:5′-diphenyl-3:3′-(3:3′-dimethoxy-4:4′-biphenylene) ditetrazolium chloride, Nitro-BT] as the final electron acceptor. An alternative series of methods is based on the use of a monotetrazolium salt, 3-(4:5-dimethylthiazolyl-2)-2:5-diphenyl tetrazolium bromide (MTT), whose formazan is capable of chelating certain metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}) to produce highly coloured products.

Superficially the results obtained with the two methods appear to be comparable, but on closer examination they are seen to differ widely. These differences have been presumed to be due to differences in the properties of the respective formazans, since that from Nitro-BT is insoluble in water and lipids, whereas the metal formazans from MTT are soluble in lipid solvents. A further postulated property of the diformazan of Nitro-BT has been described as substantivity for protein. Absence of diffusion artifact and prevention of crystallization have been attributed to binding of the diformazan to tissue proteins at the site of its production, presumably by hydrogen bonding.

Evidence was offered to show that the properties of the tetrazolium salt, rather than those of the formazan, are responsible for differences in histochemical reactions using Nitro-BT and MTT.

In spite of their relatively high redox potentials (for Nitro-BT $E_{\frac{1}{2}}$, pH 7·0, 22°, is -50 mv; for MTT it is -110 mv) tetrazolium salts are inefficient hydrogen acceptors as far as the respiratory chain is concerned. Although there is evidence that they can accept electrons at the flavoprotein level, probably only about one-tenth of those available are taken up. Alternative electron acceptors capable of passing electrons to tetrazolium salts have been employed. Of these phenazine methosulphate and menadione (K3) are most worthy of attention. Their role in dehydrogenase histochemistry was discussed.

Correlations between Results of Biochemical Methods and Cytochemical Staining Methods for Localizing Enzymes in Cells.

By S. J. Holt. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

The known structural complexities of the cell, the juxtaposition of morphological features of macromolecular dimensions, and the extraordinary dynamism of all structures microscopically observable in the living cell emphasize the importance of acquiring reliable evidence concerning the distributions of enzymic activities within *individual* cells.

Knowledge already exists concerning the distribution of enzymic activities among the classical cell fractions of tissue homogenates, but each fraction contains a mixture of similar cell fragments derived from a relatively large sample of tissue. This automatically precludes consideration of any variation in activity from fragment to fragment, and from cell to cell.

To reveal the distribution of enzymes within the constituent cells of a tissue appears to require microchemical reactions of adequate spatial re-

solving power, and which are capable of application to non-disrupted systems.

Users of methods which purport to achieve this (e.g. cytochemical staining methods) then have the responsibility of recognizing the limitations inherent in attempting to interpret their results in biochemical terms which normally apply to preparations derived from tissue masses containing large cell populations.

Even when rigorously investigated staining techniques are used, it is probable, for the present, that only *qualitative* relationships between the results of biochemical and staining approaches are capable of elucidation. Nevertheless, agreement between the results of the two approaches can enormously stimulate acceptance of both, and help to extend knowledge of cell physiology and function. When, however, results are not mutually consistent, then new levels of qualitative comparison must be sought, or the methodology of *each* approach must be re-examined.

In the light of these considerations, mutually consistent results of tissue fractionation studies (de Duve, 1959) and staining techniques (Holt, 1959) were discussed with relation to acid phosphatase, a lysosomal enzyme. Conversely, mutually contradictory results have been obtained for the distribution of esterases, which are found in the microsome fraction of rat-liver homogenates (Underhay, Holt, Beaufay & de Duve, 1956), but which appear to be lysosomal enzymes when studied by recently developed staining techniques (Holt, 1958).

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COMMUNICATIONS

(1) The Basis for the Histochemical Detection of Intracellular Glycogen.

By W. J. C. Wilkinson and J. H. Kugler (introduced by P. N. Campbell). (*Department of Anatomy, University of Sheffield, Sheffield* 10)

Opinions regarding the best technique and conditions (including fixation) for demonstrating intracellular glycogen differ widely. The histochemical demonstration of glycogen still remained capricious even after the techniques involving hydrolysis of the polysaccharide material were elaborated. This uncertainty needed to be resolved since it is of prime importance to be able to demonstrate glycogen with constancy in a given tissue. By observing glycogenolysis post mortem in guinea pig and rat livers it was found (Kugler & Wilkinson, 1959, 1960) that only part of the total tissue glycogen is detected histochemically. This fraction corresponds to the more labile acid-soluble glycogen or lyoglycogen (Willstätter & Rohdewald, 1934). The logical sequence of events was to study the histochemical picture during glycogen build-up.

Glycogenesis was studied in rabbit-liver slices which were incubated in a favourable ionic environment (Buchannan, Hastings & Nesbett, 1949). Incubation was continued for 3½ hr., samples being removed at 30 min. intervals. These specimens were each subjected to histochemical and biochemical assay. The total glycogen content of the material was estimated by the anthrone method (Seifter, Dayton, Novic & Muntwyler, 1950) and a determination was also made of the acid-soluble variety (Bloom, Lewis, Schumpert & Shen, 1951). For the histochemical detection of glycogen the tissue was stained by Gomori's hexamine silver technique, the periodic acid–Schiff method or with Best's carmine. Precise correlation between total glycogen content, the acid-soluble glycogen fraction and histochemically demonstrable glycogen could then be made. Normal cell cytology was maintained throughout the greater part of the incubation period, but when glycogenolysis exceeded glycogenesis the cells had assumed a hydropic appearance. The histochemical picture was then compared with the known amounts of both the bound and acid-soluble glycogen fractions. Thus from two widely differing experiments that involved the breakdown and synthesis of glycogen the same conclusion was reached, namely that it is only the acid-soluble fraction of tissue glycogen that is demonstrated by these histochemical techniques.

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Buchannan, J. M., Hastings, A. B. & Nesbett, F. B. (1949). *J. biol. Chem.* **180**, 435.
Kugler, J. H. & Wilkinson, W. J. C. (1959). *J. Histochem. Cytochem.* **7**, 398.
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Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
Willstätter, R. & Rohdewald, M. (1934). *Hoppe-Seyl. Z.* **225**, 103.

(2) The Histochemical Demonstration of Steroid 3β-ol dehydrogenase and Diphosphopyridine Nucleotide Diaphorase in the Adrenal Cortex.

By I. M. P. Dawson, J. Pryse-Davies and Irene M. Snape (introduced by P. N. Campbell). (*Vincent Square Laboratories of Westminster Hospital, London, S.W.* 1)

The localization in the adrenal cortex of acid and alkaline phosphatase, esterase and succinic dehydrogenase is fairly well agreed (Symington, Duguid & Davidson, 1956; Dawson, Pryse-Davies & Snape, 1960; Pryse-Davies, Dawson & Snape, 1960), but yields no information on sites of specific hormone synthesis.

Certain enzymes have specific roles in hormone synthesis; steroid 3β-ol dehydrogenase, necessary for the conversion of dehydro*epi*androsterone into androstenedione and pregnenolone into progesterone, has been demonstrated histochemically by Wattenberg (1958) using dehydro*epi*androsterone as substrate and diphosphopyridine nucleotide (DPN) as hydrogen acceptor. The reduced DPN (DPNH) is oxidized to DPN under the influence of DPN diaphorase, the hydrogen being transferred to a ditetrazolium salt, 2:2′-di-*p*-nitrophenyl-5:5′-diphenyl-3:3′-(3:3′-dimethoxy-4:4′-biphenylene) ditetrazolium chloride, Nitro-BT, which is reduced to a coloured formazan. DPN diaphorase is concerned in this demonstration; we studied its distribution by the method of Scarpelli, Hess & Pearse (1958) using DPNH as substrate. The hydrogen is transferred via DPN diaphorase to a monotetrazolium salt, 3-(4:5-dimethylthiazolyl-2)-2:5-diphenyl tetrazolium bromide (MTT); the formazan produced chelates with cobaltous chloride. We also adapted Wattenberg's (1958) technique to use pregnenolone as substrate instead of dehydro*epi*androsterone.

Fresh human and rat adrenals were sectioned immediately after operation, frozen onto a microtome chick using an acetone–carbon dioxide mixture and serially sectioned in a cryostat at −17°. Duplicate sections were extracted in acetone prior to staining and full controls performed in all cases.

We found steroid 3β-ol dehydrogenase in humans in the zona glomerulosa and outer zona fasciculata; results were identical using either dehydro*epi*androsterone or pregnenolone as substrate. DPN diaphorase has a generalized distribution throughout the cortex, most marked in the inner zona fasciculata and zona reticularis. This suggests that the conversion of pregnenolone to progesterone and dehydro*epi*androsterone to androstenedione occurs in the outer cortex.

The presence of lipid in the adrenal cortex is an additional complicating factor. The formazan formed from Nitro-BT is lipid-insoluble and its localization is therefore not interfered with. The formazan formed from MTT is lipid-soluble and therefore liable to diffusion artefact: this can be avoided by prior extraction of sections in acetone. Experiments show that the enzyme is not present in lipid and acetone does not destroy its activity.

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Pryse-Davies, J., Dawson, I. M. P. & Snape, I. M. (1960). *J. Path. Bact.* (in the Press).
Scarpelli, D. G., Hess, R. & Pearse, A. G. E. (1958). *J. biophys. biochem. Cytol.* **4**, 747.
Symington, T., Duguid, W. P. & Davidson, J. N. (1956). *J. clin. Endocrinol.* **16**, 580.
Wattenberg, L. W. (1958). *J. Histochem. Cytochem.* **6**, 225.

(3) Histochemistry and Significance of the Chromatoid Body in Spermatogenesis of the Grass Snake, *Natrix natrix*.

By B. N. Sud (introduced by P. N. Campbell). (*Cytological Laboratory, Department of Zoology and Comparative Anatomy, University of Oxford*)

The chromatoid body in *Natrix* first appears during the growth of the primary spermatocyte and is also seen in the secondary spermatocyte and late spermatid, but it is absent at metaphases of both the maturation divisions, in the early spermatid, and during the ripening of the spermatozoon. It does not make any visible contribution to the final make-up of the spermatozoon.

The chromatoid body is homogeneous, gives a low phase-change, and is not stained vitally by neutral red or Janus green. In fixed preparations it stains intensely with basic as well as acid dyes. There is no deoxyribonucleic acid in the chromatoid body. Its intense basiphilia is due mainly to its high contents of ribonucleic acid, but probably also in part to acid groups in protein.

The acidophilia of the chromatoid body indicates the presence of basic proteins. It gives an intense positive Sakaguchi reaction (Baker, 1947). After deamination it gives a negative Sakaguchi reaction but there is no change in its intense acidophilia. It is clear that the acid dyes must be reacting with basic groups other than amino groups.

It gives a negative result with the alkaline fast green reagent of Alfert & Geschwind (1953), suggesting the absence of histones. Protamines would be removed during the treatment with hot trichloroacetic acid for the removal of nucleic acids in this technique (Alfert, 1956). The negative result with alkaline fast green suggests that the arginine occurs mainly as a component of protamine rather than histone.

There is little or no tyrosine in the chromatoid body. Tests for lipid, carbohydrates, alkaline phosphatase and calcium give negative results.

The evidence suggests that the chromatoid body consists mainly of ribonucleic acid and of proteins with abundant acidic and basic groups.

Protamines consisting predominantly of arginine residues are known to replace the histones of chromatin during spermatogenesis. In *Natrix* the chromatoid body reappears during spermateleosis at the stage when this characteristic change in nucleoprotein of the spermatid is believed to take place. The chromatoid body in *Natrix*, like the sperm nucleus, is characterized by the presence of basic proteins rich in arginine and absence of histones. The abundance of ribonucleic acid and proteins in the chromatoid body may perhaps indicate that it acts as a centre for protein synthesis. It is tentatively suggested that the function of the chromatoid body is to provide basic proteins for the final maturation of the chromatin in the nucleus of late spermatids.

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Baker, J. R. (1947). *Quart. J. micr. Sci.* **88**, 115.

(4) The Influence of Sex Hormones on Melanin Pigmentation of the Skin.

By R. S. Snell (introduced by P. N. Campbell). (*Department of Anatomy, Medical School, King's College, Newcastle upon Tyne* 1)

In this investigation pure red and pure black guinea pigs were used and skin sheets as well as vertical skin sections were studied. Skin samples were removed from the ear, anterior abdominal wall, areola and sole of foot. Most of the samples were left intact but a few were split using the technique of Billingham & Medawar (1951), as modified by Szabó (1955). All the samples were then processed under exactly identical conditions using the dihydroxyphenylalanine reaction.

In the female guinea pigs following ovariectomy the melanin content of the melanocytes was reduced and many of the melanocytes became smaller in size and had shortened dendritic processes. Some melanocytes had a deformed appearance, which varied from a minor irregularity of the cell contour to an advanced degree of cell collapse with fragmentation of the dendritic processes. A significant reduction of the melanocyte count occurred only in the areola and this was accompanied by a reduction in the amount of free melanin.

In the ovariectomized animals which received oestrogen (oestradiol monobenzoate, 0·1 mg./day, intramuscularly for 5 days a week for 4 weeks), the amount of melanin was increased both inside and outside the melanocytes in all the regions. The greatest increase occurred in the areola where it was mainly due to a large increase of free melanin. No statistically significant changes in the counts were produced in any of the skin regions.

In the ovariectomized animals which received progesterone (0·4 mg./day for 5 days a week for 4 weeks) the general effect produced was similar but less than that which occurred after oestrogen treatment.

In the ovariectomized animals which received oestrogen and progesterone together in the same doses as when administered separately, the stimulating effect on melanogenesis was very much greater than when either hormone was given alone. The two hormones produced their greatest effect on the melanocytes in the skin of the areola. No statistically significant changes in the melanocyte counts were produced in any of the skin regions.

In the male guinea pigs orchidectomy resulted in a moderate increase in melanogenesis in all the regions examined. The administration of testosterone (testosterone propionate, 2 mg./day for 5 days a week for 4 weeks) to intact and orchidectomized male guinea pigs produced changes which were not clear-cut. In both groups of animals melanogenesis was stimulated in the sole of the foot, but in the orchidectomized animals inhibitory changes were observed in the skin of the anterior abdominal wall.

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(5) Histochemical Changes in Mouse Skeletal Muscle associated with *Trichinella spiralis* Infection.

By E. B. Beckett (introduced by P. N. Campbell). (*Department of Histology, University of Liverpool*)

Five to seven days after infection of a mammalian host, young *Trichinella spiralis* larvae penetrate the intestinal wall, and are carried by the blood stream to skeletal muscle. Here, each larva enters a muscle fibre, where it grows to about ten times its original size, and coils up. The affected fibre swells and degenerates into a semi-fluid 'breis', its nuclei enlarge and multiply, and it acquires a collagenous capsule when the worm is fully grown.

The disintegration of infected muscle fibres was studied in the gastrocnemii of mice infected with *T. spiralis* (kindly supplied by Professor W. E. Kershaw, Liverpool School of Tropical Medicine) and killed 5–28 days later, i.e. during the intramuscular growth period of the larvae.

Some pieces, fixed in trichloroacetic acid–ethanol and embedded in paraffin, were used to study protein-bound amino groups using the technique of Weiss, Tsou & Seligman (1954), and also to show sulphydryl groups with the dihydroxydinaphthyldisulphide method. Technical difficulties were encountered with the latter method, and these were discussed. From other pieces, fixed briefly in cold neutral formal saline, frozen sections were cut, and were used to demonstrate aminopeptidase by the technique of Nachlas, Crawford & Seligman (1958).

Normal muscle fibres show a moderate reaction for both sulphydryl and amino groups. This is diminished in fibres harbouring larvae, whilst adjacent, undamaged, fibres give a normal reaction. The larvae themselves show a concentration of both groups which is the same as, or rather greater than, that in the surrounding muscle 'breis'. Within each worm, the sites of strongest reaction are the genital primordium, and the cells composing the 'cell body' around the oesophagus.

Normal muscle fibres contain no histochemically demonstrable aminopeptidase although mast cells, blood vessels, and nerves in the connective tissue give a positive reaction. *T. spiralis* larvae and the damaged muscle substance around them show considerable aminopeptidase activity.

These histochemical observations suggest that *T. spiralis* larvae cause chemical breakdown, and probably depletion, of host muscle proteins, and it is possible that aminopeptidase is involved. It is known that the larvae can incorporate labelled amino acids administered to the host (Stoner & Hankes, 1955; Hankes & Stoner, 1958), although it is not certain that these amino acids are first incorporated into host protein. Lewert & Lee (1954) showed that *Trichinella* larvae possessed no tryptic activity.

Hankes, L. V. & Stoner, R. D. (1958). *Exp. Parasitol.* **7**, 92.
Lewert, R. M. & Lee, C. L. (1954). *J. infect. Dis.* **95**, 13.
Nachlas, M. M., Crawford, D. T. & Seligman, A. M. (1958). *J. Histochem. Cytochem.* **5**, 264.
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Weiss, L. P., Tsou, K. C. & Seligman, A. M. (1954). *J. Histochem. Cytochem.* **2**, 29.

(6) The Functional Differentiation of β Cells of Chick Embryonic Pancreas.

By T. A. I. Grillo* (introduced by P. N. Campbell). (*Anatomy School, University of Cambridge and St Mary's Hospital, London, W.* 2)

The evidence for the initial secretion of insulin, by the islet cells of developing chick embryos, has been based largely on the histological study of embryonic pancreases. Villamil (1942) reported that β cells become granulated on the 12th day. Lièvre (1957) did not find granulation until the 17th day. Hard (1944), who studied the islet cells of the rat embryo, pointed out the difficulties of recognizing cell type in early stages of differentiation of islet cells.

In the present investigation, the functional differentiation of β cells, the pancreas has been studied by both histochemical and biochemical methods. The results are compared with the histological picture at various stages of development.

The histochemical methods used aim at demonstration of sulphydryl groups present in the insulin molecule. Both the dihydroxydinaphthyl disulphide method of Barrnett & Seligman (1952) and the mercury orange technique of Bennett & Watts (1958) were used. Sulphydryl groups were demonstrated in embryonic β cells from the 16th day onwards by the Bennett technique and from the 14th day onwards by the Barrnett method.

The new histochemical technique of Schiebler & Schiessler (1959) is more specific for the demonstration of insulin in sections of the pancreas. Positive reactions were obtained in sections of embryonic pancreases of 14 and 15 days old by this technique. Insulin could not be demonstrated in the pancreas of the 11-day chick embryo.

Extracts of embryonic pancreases were made with trichloroacetic acid and acid–ethanol. Bioassays of the extracts were done by the rat diaphragm method of Randle (1956) except that D-[^{14}C]glucose was used in the medium and the radioactivity of the glycogen obtained was measured. Only the extracts from embryos of from 12-days old upwards showed insulin activity. The conclusion therefore is that the formation of insulin in the β cells begin on the 12th day of incubation. An extract of the yolk of eggs was found to have no insulin activity.

Finally, alloxan was found to have no effect on the β cells of chick embryonic pancreases inasmuch as the sulphydryl groups in the cells could still be stained by the Barrnett technique.

* In receipt of a Research Scholarship from the Federal Government of Nigeria.

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(7) The Calcification Mechanism as exemplified by a Histochemical Study of Avian Medullary Bone.

By D. A. STRINGER and T. G. TAYLOR. (*Department of Physiological Chemistry, The University, Reading*)

Medullary bone is produced in female birds at the onset of sexual maturity, and is continually resorbed and reformed according to the demands of egg-shell formation (Bloom, Bloom & McLean, 1941; Clavert, 1948). Histological and histochemical examination reveals differences in the organic matrix and in the degree of calcification between the established cortical bone, and the medullary bone.

Histological examination of the decalcified material shows that the collagen fibres of medullary bone are loosely packed, that the osteocytes are large and vesicular, and that Haversian canals are absent. Microradiographic techniques confirm the latter observation and show that there is less bone salt deposited in this organic matrix than in cortical bone.

Histochemical examination, again using decalcified material, reveals that the chemical nature of the protein matrix of the two types of bone is identical as exemplified by the coupled tetrazonium, the hydroxynaphthaldehyde, and the ninhydrin–Schiff reactions. Differences were found in the carbohydrate histochemistry; on staining with mucicarmine, dialysed iron, alcian blue, or periodic acid–Schiff, medullary bone was found to give a more intense reaction than cortical bone. These differences could be explained either by (*a*) differing concentrations of acidic and neutral mucopolysaccharides between the two types of bone, or (*b*) differing relative amounts of these mucopolysaccharides, or (*c*) a differing mode of combination with the protein matrix.

Toluidine blue staining shows that cortical bone is γ metachromatic, whilst medullary bone exhibits β metachromasia. It is possible to diminish or eliminate this metachromasia with varying concentrations of metal ions using the technique of Simkiss & Tyler (1959) in which it is assumed that the metal ion and the stain are competing for the same organic substrate. The presence of the metal ion in the substrate can be demonstrated by forming a coloured salt (e.g. CoS). The latter indicates that medullary bone contains more acidic groups than cortical bone.

These histochemical and microradiographic observations can be compared with those of Vincent (1954) who studied the changes which occur in a single osteon during calcification. It is concluded that acid mucopolysaccharides play an essential although unknown role in the initiation of calcification, and that medullary bone forms an ideal tissue for studying this process, and is to be preferred to cartilage, which is the material used by most biochemists.

Bloom, W., Bloom, M. A. & McLean, F. C. (1941). *Anat. Rec.* **81**, 443.
Clavert, J. (1948). *Bull. Biol. Fr. Belg.* **82**, 290.
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(8) Sulphate Metabolism in Epithelia.

By D. K. WATKINS and B. SPENCER. (*Department of Biochemistry, University of Wales, Cardiff*)

Following the injection of $Na_2{}^{35}SO_4$ into animals and subsequent radioautography of tissue sections it is normally assumed that mucopolysaccharide sulphates are present in those areas of the sections which show both metachromatic staining and radioactivity. From evidence of this type it would appear that the goblet cells of tracheal epithelia of rodents are active sites of mucopolysaccharide synthesis since they show radioactivity first in the supranuclear region and later in the mucous contents of the thecae (Jennings, 1958). Tracheal epithelia can be easily removed from the underlying connective tissue (Spencer, 1958), and it would seem to be a suitable tissue in which to investigate the mechanism of sulphation in the synthesis of mucopolysaccharide sulphates.

The epithelia of various slaughter-house animals were examined microscopically and that of the sheep was chosen for further study on the basis of its comparatively high number of goblet cells and liberal mucous secretion. In the presence of adenosine triphosphate, Mg^{2+} and $Na_2{}^{35}SO_4$, a particle-free preparation of sheep tracheal epithelium cells synthesized adenosine 3′-phosphate 5′-sulphatophosphate, which was characterized electrophoretically, chromatographically, spectroscopically and by the ability to donate its sulphate group to *p*-nitrophenol in the presence of phenolsulphokinase. The sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate was, in part, transferred to endogenous acceptors by various endogenous transferases in the tracheal epithelium preparation. The chromatographic pattern of the radioactive sulphated endogenous compounds was identical to that shown by rat liver and guinea-pig duodenum but the relative intensities of the various radioactive spots were different. The tracheal epithelium extract contained sulphokinases which were capable of transferring sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to various phenols, steroids, alcohols, aromatic amines and hydroxylated γ-pyrones.

The ability of epithelia to incorporate $^{35}SO_4^{2-}$ into mucopolysaccharide material was studied by incubating tissue extracts in a medium which allowed the production of adenosine 3′-phosphate 5′-sulphatophosphate. The supernatant of the incubation mixture after boiling was chromatographed using *iso*butyric acid–aqueous 0·5N-NH_3 (5:3, v/v) and the radioactivity at the origin was measured and attributed to sulphated mucopolysaccharides (Suzuki & Strominger, 1960). In this respect, sheep-tracheal epithelium had only approximately 5% of the activity shown by guinea-pig-tracheal epithelium and guinea-pig colon pieces, although the production of adenosine 3′-phosphate 5′-sulphatophosphate was of the same order in all three tissues. The sulphate-incorporating activity of each tissue was localized in the soluble material of 0·25M-sucrose homogenates and sonicates of the particulate material were inactive (cf. Suzuki & Strominger, 1960). Crude ox potassium chondroitin sulphate stimulated the incorporation of $^{35}SO_4^{2-}$ into mucopolysaccharide material by 700% in the case of guinea-pig colon extracts but no stimulation was observed with the tracheal epithelium extracts from either guinea pig or sheep.

Jennings, M. A. (1958). *Quart. J. exp. Physiol.* **43**, 60.
Spencer, B. (1958). *J. Histochem. Cytochem.* **6**, 105.
Suzuki, S. & Strominger, J. L. (1960). *J. biol. Chem.* **235**, 257.

(9) Differences in the Utilization of Labelled Precursors for the Synthesis of Deoxyribonucleic Acid in Cell Nuclei and Psittacosis Virus.

By S. R. Pelc* and T. T. Crocker. (*Cancer Research Institute, University of California, Medical Center, San Francisco, U.S.A.*)

Psittacosis virus grows in colonies in the cytoplasm of infected HeLa cells and the incorporation of labelled precursors into the deoxyribonucleic acid (DNA) of the virus can be observed by means of radioautography. HeLa cells were grown in Leighton tubes containing cover slips, labelled medium was added at various times after infection and radioautographs prepared after washing and fixation.

Cell counts and the increase in the number of labelled nuclei show that the duration of the mitotic cycle in HeLa cells is 26 hr.; DNA synthesis takes 6·5 hr. In infected cells the generation time and time of synthesis are doubled. HeLa cells labelled for 23 hr. with [^{3}H]thymidine show a loss of 40% in the average number of grains per nucleus 3 hr. after transfer to unlabelled medium. This loss is not affected by infection. Subsequently the decrease is compatible with the generation time. If such cultures were infected with psittacosis virus after washing, no labelling of the DNA of the viral inclusions was observed. Feulgen photometry shows that 24 hr. after infection the amount of DNA of a viral colony is on an average twice that of the nucleus. Preparations labelled 3–20 hr. after infection with [^{3}H]cytidine or [^{3}H]uridine and treated with ribonuclease before radioautography show approximately 3 times the number of photographic grains per colony as the nuclei; the strength in the two sites is not correlated.

Uptake of [^{3}H]thymidine in viral colonies amounts to less than 5% of that in the nuclei; more heavily labelled colonies are usually associated with heavier nuclei. The base ratios of the DNA of psittacosis virus are within the normal range and the low utilization of [^{3}H]thymidine cannot be due to lack of thymine in the DNA. It can be explained if it is assumed that thymidilic acid is normally synthesized from uridilic acid by the pathways suggested by Friedkin (1959) and by Humphreys & Greenberg (1958). Thymidine as such nowhere appears in this scheme and therefore can not be regarded as a natural precursor in the synthesis of DNA. The utilization of [^{3}H]thymidine will depend on: (*a*) the presence and activity of enzymes able to phosphorylate thymidine, and (*b*) on the ability of this compound to reach the pathway. It is concluded that DNA synthesis of psittacosis virus is not directly coupled with nuclear DNA synthesis and that the processes (*a*) and (*b*) are available to cell nuclei but not for viral growth.

Friedkin, M. (1959). In *The Kinetics of Cellular Proliferation*. Ed. by Stohlman, F. New York: Grune and Stratton.
Humphreys, G. K. & Greenberg, D. M. (1958). *Arch. Biochem. Biophys.* **78**, 275.

(10) Histochemical and Electron Microscopical Observations on the Acroblast of *Helix aspersa*.

By S. Bradbury, J. T. Y. Chou and G. A. Meek (introduced by P. N. Campbell). (*Department of Human Anatomy, Oxford University and the Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, Oxford University*)

The acroblast is a cytoplasmic inclusion found in the spermatocytes and spermatids of many animals; it is first apparent in the primary spermatocyte,

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where it seems to originate from the fusion of many smaller structures having a rod-like appearance in optical section. The acroblast is not apparent during the meiotic divisions of the spermatocytes but again becomes very prominent in the spermatid stage. Previous workers have shown it to be responsible for the secretion of the acrosomal cap which covers the head of the sperm. After performing this function the remnants of the acroblast pass to the tail region of the sperm and are ultimately dispersed and cast off with the residual cytoplasm of the spermatid into the lumen of the seminiferous tubule.

Optical studies on both living and fixed material have confirmed the views of previous authors that the acroblast is a duplex body, having an outside sheath or 'externum' often shaped like a deep cup, enclosing an inner substance (the 'internum') of differing reactions. Histochemical methods have shown the externum to contain protein, much phospholipid, and appreciable quantities of alkaline phosphatase. The internum contains much neutral mucopolysaccharide or mucoprotein. Electron microscopy has revealed the externum to be composed of many paired non-granular membranes, associated with vesicles, whilst the internum is characterized by the presence of 'compound vacuoles'. These are small vesicles enclosed within a limiting membrane; other aggregates of small vesicles, with no associated membrane, are often also seen in this region.

It is suggested that the lipoprotein membranes of the externum are responsible for the segregation of the 'compound vacuoles' of the internum, and it is these which may comprise the precursors of the acrosome.

Similar bodies have been noted by other authors, e.g. Sotelo and Porter in rat oocytes, and further work may reveal a general correlation between the presence of compound vacuoles and mucoprotein secretion. The present work is a further illustration of the importance of correlating observations on the living cell, with the results of histochemical techniques and with electron microscopical observations at every stage.

(11) The Uptake of [^{3}H]Leucine into Human Lymphocytes *in vitro*.

By E. H. Cooper.* (*Medical Unit, St Mary's Hospital, London, W. 2*)

One or two lymph nodes were obtained from the chest during cardiac surgery; the nodes were chopped and incubated for 1 hr. at 37·5° in Hanks's medium (Hanks, 1948) with 50% autologus serum; the cells were then filtered through tantalum gauze and suspended in Hanks's medium with 20% serum. The uptake of DL-[^{3}H]leucine (3 μc/ml., 100 mc/m-mole) was studied at 37·5°; after incubation the cells were spun down and slides prepared, dried in air and extracted with methanol. Kodak stripping film (AR 10) was applied and exposed for 2–3 weeks. The uptake of leucine was measured by counting the grains over individual cells. The large and medium lymphocytes (lymphoblasts) had approximately twice the uptake of leucine per square micron compared to the small lymphocytes. More than 99% of the small lymphocytes had a fairly uniform rate of uptake of leucine, and less than 1% were very strongly labelled relative to the general population of these cells. The lymphoblasts are metabolically a heterogeneous population, individual cells showing considerable variation in the uptake of leucine, some being labelled 5–6 times greater than the average labelling of the particular cell type.

Inhibition of the uptake of leucine into lymphoblasts and small lymphocytes *in vitro* was observed on addition to the medium of *p*-fluorophenylalanine (5 mM), ethionine (3 mM), 2:4-dinitrophenol (5 mM) or methionine sulphoximine (2 mM), the latter inhibition being reversed by glutamine (0·5 mM).

Peripheral blood leucocytes were separated by precipitation of erythrocytes with dextran, washed and incubated at 37·5° in Hanks's 20% serum with DL-[^{3}H]leucine (3 μc/ml.). Radioautographs of these cells demonstrated that the small lymphocytes have a higher uptake of leucine than the polymorphonuclear leucocytes. The monocytes and large lymphocytes were the most heavily labelled cells. Daily estimation of the uptake of leucine under standard conditions *in vitro* by the blood leucocytes of the same subject showed little variation.

The author is grateful to Mr L. Bromley, Thoracic Surgeon, St Mary's Hospital, for providing lymph nodes specimens from his patients.

Hanks, J. H. (1948). *J. cell comp. Physiol.* **31**, 235.

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(12) Some Aspects of Amino Acid Metabolism in the Housefly, *Musca domestica*.

By G. M. Price. (*Biochemistry Department, Agricultural Research Council, Pest Infestation Laboratory, Slough, Bucks*)

The amino acids, α-alanine, β-alanine, arginine, aspartic acid, glutamic acid, glutamine, glycine,

leucine, lysine, methionine, methionine sulphoxide, phenylalanine, proline, serine, taurine, tyrosine and valine, have been identified in ethanol extracts of haemolymph and of whole adult houseflies, by one- and two-dimensional paper chromatography. Those amino acids which are in equilibrium with the Krebs tricarboxylic acid cycle were estimated quantitatively, essentially in the manner described by Yemm & Cocking (1955). Aspartic and glutamic acids were more concentrated in the tissues of the fly than in the haemolymph, while the concentrations of α-alanine, glutamine and proline were approximately the same in both tissues and haemolymph.

After flies had been injected with [2-^{14}C]acetate, labelled α-alanine, aspartic acid, glutamic acid, glutamine and proline were identified in extracts of haemolymph and of whole flies. The result confirmed the findings of Winteringham & Harrison (1956). The specific activities of these amino acids were measured at intervals up to 3 hr. after injection of the flies with [2-^{14}C]acetate. It was found that the specific activities of α-alanine, aspartic acid and glutamic acid were maximal 60 min. after the flies were injected, while those of glutamine and proline were maximal after 80 and 90 min. respectively. The nature of the specific activity/time curves of the amino acids (Zilversmit, Entenman & Fishler, 1943), suggested that, in the fly, glutamic acid is a precursor of glutamine, but not of proline. The latter observation is surprising because, in a number of widely different organisms, it has been shown that proline can be derived from glutamic acid (Ehrensvärd, 1955).

The rate of production of $^{14}CO_2$ by 'labelled' flies was maximal over the period 50–70 min. after they had been injected and by the end of this period over 95% of the [2-^{14}C]acetate originally present in the fly had been metabolized.

Hydrolysis of the protein fraction from 'labelled' flies showed that it contained labelled α-alanine, aspartic acid, glutamic acid and proline. A number of unlabelled amino acids were also identified in the hydrolysate, but β-alanine and methionine sulphoxide were absent, indicating that these two amino acids are not constituents of 'housefly' protein.

Glutamic–alanine transaminase and glutamic–aspartic acid transaminase were demonstrated in flight muscle sarcosomes, but were absent from the haemolymph.

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Winteringham, F. P. W. & Harrison, A. H. (1956). *Nature, Lond.*, **178**, 81.
Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

(13) Incorporation of [2-^{14}C]Acetate into Acetylcholine of the Adult Housefly *in vivo* under Conditions of Rest, Activity and Insecticidal Action.

By F. P. W. Winteringham and A. Harrison. (*Biochemistry Department, Agricultural Research Council, Pest Infestation Laboratory, Slough, Bucks*)

The [^{14}C]acetylcholine formed *in vivo* following the intrathoracic injection of [2-^{14}C]acetate into the adult housefly *Musca domestica* has been separated by paper chromatography from the other labelled compounds formed and then assayed radiometrically. Maximum specific radioactivity of the acetylcholine fraction was rapidly achieved within 15 min. On the basis of the total acetylcholine of the head tissues assayed pharmacologically (Lewis & Smallman, 1956) isotopic equilibrium with the injected acetate was never achieved although the latter was apparently present in the tissues in excess for considerably longer than 15 min. (G. M. Price, personal communication).

Continuous *cyclo*propane anaesthesia lowered the rate of formation of [^{14}C]acetylcholine and also the overall metabolism of acetate. Anticholinesterases, on the other hand, appeared to 'uncouple' acetylcholine synthesis in the sense that the overall metabolism of [^{14}C]acetate and respiration were increased while formation of [^{14}C]acetylcholine was considerably reduced. The effect of the insecticide malathion on formation of [^{14}C]acetylcholine was partly reversed on injecting pyridine-2-aldoxime methiodide together with atropine but unlike the case of di*iso*propyl phosphorofluoridate there was no reversal of the outward signs of poisoning. There was no evidence that the insecticide dieldrin directly affected the metabolism of acetylcholine *in vivo*. Some of these data are consistent with the 'synaptic vesicle' hypothesis recently examined by Whittaker (1959).

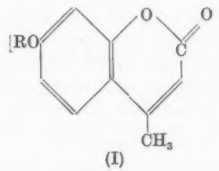
Lewis, S. E. & Smallman, B. N. (1956). *J. Physiol.* **134**, 241.
Whittaker, V. P. (1959). *Biochem. J.* **72**, 694.

(14) Properties of Some Derivatives of 4-Methylumbelliferone.

By D. H. Leaback. (*Department of Biochemistry, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

Mead, Smith & Williams (1955) introduced methylumbelliferyl β-D-glucuronide as a substrate for the fluorimetric assay of β-glucuronidase; Levvy, Hay & Marsh (1957), however, considered the method unsuitable for general use. Leaback & Walker (1960) showed that methylumbelliferyl

N-acetyl-β-D-glucosaminide had advantages over the corresponding *p*-nitrophenyl derivative as a substrate for *N*-acetyl-β-glucosaminidase. In order to assess some further methylumbelliferyl derivatives (I) as potential enzyme substrates, certain properties of these compounds have now been compared with those of the *N*-acetyl-β-D-glucosaminide.



(I)

Methylumbelliferyl acetate, phosphate, sulphate β-D-glucoside, β-D-galactoside, and β-D-glucuronide were synthesized by modifications of standard procedures, taking special care to remove traces of residual free methylumbelliferone from the preparations. Methylumbelliferyl acetate showed extreme lability under basic conditions and for this reason is unlikely to find application as an enzyme substrate; the other methylumbelliferyl derivatives showed stabilities at pH 4·9 and 10·3 comparable to those of the *N*-acetyl-β-D-glucosaminide (Leaback & Walker, 1961). The phosphate and sulphate and were readily soluble in water, but the β-D-galactoside was markedly less soluble than the other β-glycosides; this will limit the range of usefulness of the β-D-galactoside as an enzyme substrate.

All the methylumbelliferyl derivatives (I) had absorption bands at about 280 and 315 mμ, which varied in intensity according to the nature of the substituent *R*. Excitation and fluorescence spectra of the phosphate and sulphate (cf. Mead *et al.* 1955) were similar in form to, but lower in intensity than, those of the β-glycosides. The excitation spectra as measured on the Aminco spectrofluorimeter were distorted by instrumental factors, but were valuable in excluding the possibility of the compounds exhibiting radically different excitation and absorption spectra (cf. Förster, 1950); the absorption spectra were thought to provide a better basis for the selection of primary filters for the Locarte fluorimeter.

Results indicated that methylumbelliferone could be estimated in the presence of excess methylumbelliferyl phosphate, sulphate, β-D-glucoside, β-D-galactoside or β-D-glucuronide using the spectrophotometric and fluorimetric techniques described previously (Leaback & Walker, 1960, 1961); these compounds should therefore prove satisfactory as substrates for enzyme estimations.

The author is indebted to Professor R. T. Williams for the use of the spectrofluorimeter, to Dr P. G. Walker for advice, to Dr F. B. Anderson for some β-D-glucuronide and to London University and the Nuffield Foundation for research grants.

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(15) The Effect of 2-Dimethylaminoethanol on the Metabolism of Cerebral Phospholipids

By G. B. Ansell and Sheila Spanner. (*Department of Experimental Psychiatry, The Medical School, Birmingham*, 15)

Low concentrations (40–80 μg./kg. fresh wt.) of 2-dimethylaminoethanol are present in brain tissue (Honegger & Honegger, 1960) and the base is of interest as a possible precurser of choline (Venkataraman & Greenberg, 1958). In view of the relative ease with which 2-dimethylaminoethanol enters the brain (Groth, Bain & Pfeiffer, 1958) and its conceivable action as a cerebral stimulant (Pfeiffer, 1959) experiments have been carried out on its effect on the metabolism of phospholipids.

Within an hour of an intraperitoneal injection of 150 mg./kg. of the base into the rat there was an apparent large increase in the phosphorylcholine level of brain tissue. Closer examination of chromatograms on which the acid-soluble phosphate esters had been separated showed, however, that another phosphate ester was present. This ester, which was difficult to separate by chromatography from phosphorylcholine, has been identified as phosphoryldimethylaminoethanol by comparison with synthetic material and by isotope-dilution experiments. The level of this ester reached its peak (0·5 μmole/g. fresh wt.) in the brain 1–2 hr. after injection of the base, falling to a level of 0·2 μmole/g. fresh wt. within 8 hr. whilst phosphorylcholine levels were unchanged. The level of phosphoryldimethylaminoethanol depended on the amount of base injected and no evidence was obtained of its presence as a normal constituent of brain tissue. The ester has been isolated from a choline-requiring strain of *Neurospora crassa* (Wolf & Nyc, 1959).

Experiments with [^{32}P]orthophosphate showed that 2-dimethylaminoethanol significantly increased the uptake of isotope into brain phosphatidyl choline *in vivo* which is in agreement with the observations on liver by Artom & Cornatzer (1948). Of further interest was the slight stimulatory effect of 2-dimethylaminoethanol on the

phosphate uptake by brain phosphatidic acid *in vivo*. Hokin & Hokin (1959) have shown that phosphate exchange by this phospholipid *in vitro* is stimulated by low concentrations of acetylcholine, and 2-dimethylaminoethanol is believed to raise cerebral acetylcholine levels (Pfeiffer, 1959); this has however been denied (Pepeu, Freedman & Giarman, 1960). In the present study there was a strong indication that 0·25 mM-2-dimethylaminoethanol increased the formation of radioactive phosphatidic acid when brain dispersions were incubated for 1 hr. with [^{32}P]orthophosphate in the presence of eserine.

The results were discussed in relation to the possible role of 2-dimethylaminoethanol in brain metabolism.

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(16) The Fractionation of Polyanions by Long-Chain Aliphatic Ammonium Salts.

By J. E. Scott.* (*Surgical Unit, St Mary's Hospital Medical School, London, W.* 2)

Polyanions, (P^{N-}) (e.g. heparin and hyaluronic acid) are precipitated by long-chain aliphatic ammonium salts (R^+, e.g. cetylpyridinium chloride) (Scott, 1960, 1955). The concentration of R^+, [R^+], in equilibrium with the precipitate at flocculation point (i.e. the 'excess amine concentration') decreases by a factor of 10 for each increase in chain length of $2CH_2$ units. This is deducible if the neutral polyanion–R^+ complex is an 'oily' particle, and the transfer of R^+ from aqueous solution to this particle is accompanied by a liberation of free energy of $1{\cdot}2RT$ per CH_2 unit—in agreement with the value for the transfer of CH_2 from water to a detergent micelle, or to oil (Philips, 1955).

The occurrence of a sharp 'flocculation endpoint' (Scott, 1960) is accounted for on applying the law of mass action to (1),

$$P^{N-}{}_N M^+ + {}_N R^+ \overset{K}{\rightleftharpoons} P^{N-}{}_N R^+ + {}_N M^+ \qquad (1)$$

where M^+ is the inorganic counterion.

If N (the valency) = 1, there is no inflexion in the curve of ($P^{N-}{}_N R^+$)/($P^{N-}{}_N M^+$) against R^+/M^+ but as N increases an almost rectangular inflexion appears. The value of K, the equilibrium constant, determines the position of the inflexion on the axis R^+/M^+.

An increase in [M^+] incurs an increase in excess amine concentration, because of competition between M^+ and R^+ according to (1). If [M^+] is maintained constant, the excess amine concentration depends on the nature of P^{N-}. Conversely, if [R^+] is constant, [M^+] at flocculation is dependent on the nature of P^{N-}. There are therefore two possible ways of fractionally precipitating P^{N-} mixtures: by maintaining [R^+] constant and varying [M^+], or by maintaining [M^+] constant and varying [R^+].

The upper limit of [R^+] is the critical micellar concentration. If micelles are assumed to be ineffective precipitants, in comparison with R^+, and [R^+] is constant in the presence of micelles, the variables in (1) are [M^+], K, and N. A polyanion with given N and K would therefore not be precipitated by R^+ at a 'critical salt concentration', [M^+] (Scott, 1960), which would be a constant for the polyanion.

As a corollary, from (1) the plot of 1/N against log M^+ should be a straight line. This relationship was discussed in the light of the fractionation of hyaluronic acid and heparin, using cetylpyridinium chloride and inorganic salt, as performed by Laurent (Laurent, Ryan & Pietruszkiewicz, 1960; and personal communication).

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* Leverhulme Research Fellow.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **401st Meeting** *of the Society was held in the Physiology Theatre, Guy's Hospital Medical School, London, S.E.*1, *on Friday,* 16 *December* 1960, *when the following papers were presented:*

COMMUNICATIONS

(1) Biogenesis of 3:6-Anhydro-L-Galactose

By D. A. Rees. (*Chemistry Department, Edinburgh University*)

Although it was suggested as early as 1942 that the precursor of the 3:6-anhydro-L-galactose residues present in commercial agar might be L-galactose 6-sulphate (Jones & Peat, 1942), no evidence has been offered either in confirmation of this hypothesis, or for the presence of derivatives of galactose 6-sulphate in seaweeds. Moreover, there are several ways in which the anhydro sugar might arise from galactose sulphate. It could be an artifact produced in the processing of the polysaccharide; it could be formed as a result of the interconversion of sugar nucleotides (compare Hassid, Neufeld & Feingold, 1959), or it could be formed by enzyme action on an oligo- or polysaccharide containing galactose sulphate residues.

Recent work has shown that porphyran, a marine red algal polysaccharide, contains residues of both 3:6-anhydro-L-galactose (Peat, Turvey & Rees, 1961) and L-galactose 6-sulphate (Turvey & Rees, 1961). When extracts of the seaweed from which porphyran is derived are incubated with the polysaccharide, free sulphate is liberated (Peat & Rees, 1961) and synthesis of 3:6-anhydrogalactose residues occurs. It is not yet clear whether the same enzyme or a system of closely associated enzymes is responsible for these phenomena. Equimolar quantities of the two products are present in the mixture after incubation, and also at intermediate stages. The enzyme catalysing the release of sulphate is adsorbed by a small volume of calcium phosphate gel, being easily eluted by strong, but not by dilute phosphate buffer; the anhydrogalactose-synthesizing enzyme is also purified in this way.

These results provide a strong indication that L-galactose 6-sulphate is an immediate biological precursor of 3:6-anhydro-L-galactose, and further, that since the sulphatase substrate is a large molecule (Peat & Rees, 1961) the precursor units are combined in a high-molecular-weight polymer.

This work is being continued, and it is hoped to investigate also the biogenesis of 3:6-anhydro-D-galactose. The author thanks Professor Stanley Peat, Professor E. L. Hirst and Dr J. R. Turvey for their interest, and the D.S.I.R. for a Fellowship.

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(2) The Biosynthesis of Amino Sugars by Intestinal Mucosa

By C. A. Pasternak. (*Department of Biochemistry, University of Oxford*)

Mammalian intestinal mucosa synthesizes sulphated mucopolysaccharides *in vitro* (Pasternak, Kent & Davies, 1958). An initial reaction is the activation of sulphate by adenosine triphosphate to form 3′-phosphoadenosine 5′-phosphosulphate (Robbins & Lipmann, 1956; Kent & Pasternak, 1958), from which the sulphate group is transferred to mucopolysaccharides (Varandani, Wolf & Johnson, 1960). The origin of the amino sugar in such mucopolysaccharides has not yet been elucidated. In other tissues, synthesis of glucosamine 6-phosphate from hexose 6-phosphate and glutamine is a key reaction (Roseman, 1959). Colonic mucosa forms hexose 6-phosphate by virtue of its high glycolytic rate (Rosenthal & Lasnitzki, 1928), and it was of interest to determine whether the reaction with glutamine takes place.

An extract of bovine colon was prepared by homogenizing mucosal scrapings in 3–4 vol. of 0·25 M-sucrose containing 0·01 M-cysteine and 0·01 M-ethylenediaminetetra-acetic acid (pH 7·5) and centrifuging at 105 000*g* for 60 min. Incubation of the supernatant fraction (0·2 ml.) with D-glucose 6-phosphate (4 μmoles), L-glutamine (2 μmoles) and 2-amino-2-hydroxymethylpropane-1:3-diol buffer (100 μmoles, pH 7·5) in a final volume of 1 ml. resulted in the formation of glucosamine 6-phosphate at an initial rate of 3·5 μm-moles/mg. of protein/min. When glucose 6-phosphate was replaced by other hexose phosphates, the amount of glucosamine 6-phosphate synthesized, expressed as a percentage of that from glucose 6-phosphate, was as follows: fructose 6-phosphate, 92%; mannose 6-phosphate, 72%; galactose 6-phosphate, 33%; ribose 5-phosphate, 30%. Two analogues of

L-glutamine, *O*-diazoacetyl-L-serine and 6-diazo-5-oxo-L-norleucine, which were given by Parke, Davis and Co., inhibit this reaction markedly (50% inhibition at mM and 10 μM, respectively). An enzyme with similar properties was found in colonic extracts of sheep and rabbit.

A further reaction in mucopolysaccharide formation is the acetylation of glucosamine 6-phosphate by acetyl-coenzyme A (Roseman, 1959). Bovine mucosal extracts were found to catalyse this reaction also, starting either with glucose 6-phosphate and glutamine or with glucosamine 6-phosphate. A reaction mixture containing glucosamine 6-phosphate (0·8 μmole), acetyl-coenzyme A (0·3 μmole), phosphate buffer (50 μmoles, pH 7·2) and mucosal extract (0·2 ml., 1·7 mg. of protein) in 0·5 ml. gave quantitative formation of *N*-acetylglucosamine 6-phosphate (0·3 μmole) within 30 min. of incubation.

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(3) Hexitols in Foetal Sheep Blood

By H. G. Britton (introduced by A. St. G. Huggett). (*Department of Physiology, St Mary's Hospital Medical School, London, W. 2*)

When extracts of fresh foetal sheep blood were examined by conventional paper-chromatographic techniques a single spot was found in the position for sorbitol. Using a quantitative technique (Britton, 1958, 1959) the amount of sorbitol present in a number of samples was found to be of the order of 10 mg./100 ml. Recently, however, using the chromatographic system ethyl methyl ketone–acetic acid–water saturated with boric acid (9:1:1, by vol.) (Rees & Reynolds, 1958) and developing with silver nitrate (Britton, 1959) evidence was obtained that the material running in the position for sorbitol in conventional systems was not homogeneous. The composition of this sorbitol-like material was therefore further investigated by electrophoresis in basic lead acetate (Frahn & Mills, 1959) which permits a separation of all the isomeric hexitols. Development of the electrophoresis strips was carried out by a modification of the silver method of Trevelyan, Procter & Harrison (1950) in which the basic lead acetate was converted into fluoride by dipping in a solution of hydrogen fluoride (40%, 1 vol.) in acetone (10 vol.) after impregnation with silver nitrate and before treatment with alkali. Using this technique the sorbitol-like material has been shown to be a mixture of three components which have been provisionally identified as mannitol, galactitol and sorbitol.

When the placenta of the sheep is perfused previous work by Andrews, Britton & Nixon (1959) had shown that there is an accumulation of sorbitol-like material. Preliminary work would indicate that this rise in concentration is largely if not entirely, in the sorbitol fraction.

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(4) The Effect of Anoxia and Insulin on Hexose Phosphate Levels in Rat Muscle

By E. A. Newsholme and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

The levels of glucose 6-phosphate, fructose 6-phosphate, and fructose 1:6-diphosphate in trichloroacetic acid extracts of frozen muscle powder prepared from perfused isolated rat hearts, and rat hemidiaphragms, have been assayed by enzymic methods. In all experiments hearts were perfused for 15 min. with bicarbonate-buffered salt solution containing 1 mg. of glucose/ml. and diaphragms incubated in similar medium but containing 2·5 mg. of glucose/ml.

In hearts perfused aerobically without insulin, levels of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate were 22, 4·7, and 2·0 μmoles/100 g.; under anaerobic conditions without insulin, levels were changed by −20%, −22% and +63% respectively; in presence of insulin under aerobic conditions the levels were changed by +9% and +17% and +82% respectively; in the presence of insulin anaerobic conditions led to changes of −33%, −31% and +25% respectively. We conclude that anoxia in the presence or absence of insulin leads to a fall in levels of glucose and fructose 6-phosphates and to a rise in the level of fructose 1:6-diphosphate in rat heart.

In diaphragms incubated aerobically in the presence of insulin, levels of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate were 40, 10·2, and 2·4 μmoles/100 g.; under anaerobic conditions these levels changed by −27%, −30% and +33% respectively. It is con-

cluded that in rat diaphragm also anoxia leads to a fall in levels of glucose and fructose 6-phosphates and to a rise in the level of fructose 1:6-diphosphate.

We conclude from these changes that anoxia leads to an increase in the activity of phosphofructokinase (or alternatively to a diminution in the activity of fructose 1:6-diphosphatase) in heart and diaphragm muscle. These results also suggest that insulin action may lead to an increase in the activity of phosphofructokinase (or to a diminution in that of fructose 1:6-diphosphatase) in cardiac muscle.

Morgan, Randle & Regen (1959) have shown previously in the rat heart, that phosphorylation of glucose is stimulated by anoxia in the presence of insulin. The present results suggest that this effect of anoxia may be brought about by a fall in the intracellular level of glucose 6-phosphate, a known inhibitor of muscle hexokinase (Crane & Sols, 1953).

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(5) Isolation and Some Enzymic Activities of Muscle-Cell Membranes

By D. L. McCollester* and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

The paravertebral muscles of a 300 g. rat were placed in 100 ml. of 0·8 % $CaCl_2$ at 0° and minced with a high-speed Waring Blendor for 10 sec. Tissue not pouring through a plastic net of 1·5 mm. square holes was resubjected to a similar treatment. This process, repeated 3 or 4 times, reduced the muscle to 0·1–1·0 mm. lengths of muscle cell. This homogenate was briefly centrifuged, washed 4 times in 10 vol. of 0·025 M-NaCl and 2·5 mM-histidine–2-amino-2-hydroxymethylpropane-1:3-diol buffer (pH 6·8), incubated at 37° for 30 min. in 10 vol. of the wash solution plus 5 mM-$MgCl_2$, and then washed thrice with 10 vol. of the wash solution at pH 6·8 and twice at pH 7·4. Finally, the homogenate was washed once in 10 vol. of 2 μN-NaOH in doubly distilled water, centrifuged at 2400*g* for 10 min., and resuspended in 100 vol. of 2 μN-NaOH. This last procedure was repeated twice more. During this treatment the contractile proteins dissolved and along with the intracellular structure gushed from the ends of the muscle-cell fragments. Ordinary and phase-contrast microscopy showed that the final product consisted of thin structureless tubes and adherent capillaries.

* U.S. Public Health Service Research Fellow of the National Heart Institute.

Enzymic activities of the homogenate and the membranes were estimated as follows: hexokinase by the amount of glucose 6-phosphate produced in the presence of adenosine triphosphate (ATP); aldolase by the oxidation of dihydrodiphosphopyridine nucleotide in the presence of fructose 1:6-diphosphate and glycerophosphate dehydrogenase; phosphofructokinase by the oxidation of dihydrodiphosphopyridine nucleotide in the presence of fructose 6-phosphate, ATP, aldolase, and glycerophosphate dehydrogenase; phosphorylase by the inorganic phosphate produced in the presence of glucose 1-phosphate and glycogen; adenosine triphosphatase by the inorganic phosphate produced in the presence of 0·15 M-KCl, 5 mM-ATP and 5 mM-$MgCl_2$ at pH 7·4. The activities observed per gram of homogenate per hour were: hexokinase, 1·7 μmoles of glucose 6-phosphate; aldolase, 11 μmoles of fructose 1:6-diphosphate; phosphofructokinase, 4·6 μmoles of fructose 6-phosphate; phosphorylase, 490 μmoles of inorganic phosphate; adenosine triphosphatase, 280 μmoles of inorganic phosphate. Of these activities, 0·5 %, 0–2 %, 0·9 %, 0·02 % and 16 % respectively, were estimated to be associated with the membranes. We conclude that no glycolysis occurs in the membranes. Those enzymic activities which appear to be associated with the membranes are being investigated further.

(6) Some Aspects of Inosine Metabolism in Red Cells

By J. S. Wiley and R. Whittam. (*Department of Biochemistry, University of Oxford*)

Lactate production from inosine in human red cells is five times greater in isotonic sodium phosphate solution than in physiological saline medium during incubation at 37° (Lowry, Jaffe, Vanderhoff, Crook & London, 1958). To see whether this variation depends rather on the intracellular phosphate concentration, lactate production from inosine was measured in cells containing different amounts of phosphate. These were obtained by incubating washed fresh cells from 0 to 4 hr. at 37° in saline media containing 0, 10 and 40 mM-phosphate. The incubation was then continued for a further hour after the addition of inosine (13 mM). Lactate production (2·9–15·3 m-moles/l. of cells) was directly proportional both to the phosphate concentration (1·8–14·7 m-moles/l. of cells) before addition of inosine and to the mean amount of phosphate esterified (1·9–14·9 m-moles/l. of cells). The phosphate esterified during the first 8 min. was equal to the hypoxanthine produced by phosphorylysis of inosine.

To find out whether glucose utilization in fresh cells was affected by simultaneous lactate production

from inosine, the disappearance of glucose was measured in the presence and absence of inosine. Some fresh cells were preincubated for 3·5 hr. in order to increase their phosphate concentration by hydrolysis of endogenous phosphate esters (Whittam, 1958). The lactate produced from glucose in preincubated and fresh cells (2·5 and 3·5 m-moles/l. of cells/hr.) was equivalent to the glucose disappearing (1·3 and 1·8 m-moles/l. of cells/hr.); the addition of inosine (15 mM) reduced glucose utilization by 60 and 100 %, depending on the total rate of lactate production (3·7 and 6·1 m-moles/l. of cells/hr.). The metabolism of ribose 1-phosphate derived from inosine therefore suppresses the disappearance of glucose.

An esterification of endogenous adenosine mono- and di-phosphate to adenosine triphosphate occurred during incubation with inosine (Overgaard-Hansen, 1957) and we have also found that inosine monophosphate was formed but not inosine di- or tri-phosphate. The concomitant addition of ^{14}C-labelled adenine caused a net synthesis of adenosine di- and tri-phosphate (see Nakao, Nakao, Tatibana, Yoshikawa & Abe, 1959) of about 0·3 m-mole ester phosphate/l. cells/hr. between 1 and 4 hr. of incubation and AMP was also formed. These results show that inosine produces lactate proportional to the intracellular phosphate concentration and also supports the formation of adenine nucleotides from adenine.

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(7) The Effect of Stress and of Ethanol on the Lactic Acid of the Brain

By J. W. Ridge. (*Burden Neurological Institute, Stapleton, Bristol* 5)

The work of Richter and his colleagues has shown that stress causes an increase in the lactate and creatine phosphate of the rat brain (Richter & Dawson, 1948; Dawson & Richter, 1950) and a decrease in acetylcholine (Richter & Crossland, 1949). As a preliminary to a further study of the effects of stress and ethanol (separately and together) on the brain the work on lactate has been repeated and extended.

The method of stress used was that of gentle tumbling for periods up to 2 min. The rats were killed by immersion in liquid oxygen and lactate estimated by the method of Barker & Summerson (1941).

On tumbling the animals the brain lactate rose within the first minute from the normal level of 22·0 mg./100 g. of tissue (wet weight) to 36·3 mg./100 g. Further stress did not raise this figure. The brain lactate began to fall as soon as the animal was released from the stress and regained the normal level in about 4 min. These results are in agreement with Richter & Dawson (1948) who did not extend their experiments much beyond this point and who therefore concluded that the effect of such stress was transitory. The present results show that the brain lactate continued to fall for a further 3 or 4 min. to about 18 mg./100 g. and then began to increase again. It overshot the normal and went into a series of damped oscillations having a period of about 10 min. The effect lasted about 40 min.

Ethanol (3 ml.; 30 %, v/v) was administered by stomach tube. This procedure was in itself slightly stressing; the administration of 3 ml. of water caused an immediate rise to about 25 mg. of lactate/100 g., falling again to the normal in about 5 min. Thereafter there was some slight evidence for an oscillation of small amplitude about the normal. The ethanol curve showed the initial stress maximum and a secondary, small maximum after about 4 min. before the brain lactate went into a long slow oscillation of large initial amplitude (minimum 18·3 mg./100 g., maximum 35 mg./100 g.) and a period of about 35 min. Like the stress curve, the ethanol curve was asymmetrically disposed about the normal.

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(8) The Role of Cerebroside Sulphate (Sulphatide) in the Brain

By A. N. Davison. (*Guy's Hospital Medical School, London, S.E.* 1)

Hitherto, suggestions for the role of sulphatide in the brain have only been tentative, for knowledge of sulphatide metabolism and distribution has been very limited. In the present work sulphatide metabolism in the brains of young rats has been compared with that in adult rats. Doses of [^{35}S]-sulphate proportional to body weight were given to rats of various ages. Ten times as much radioactivity was incorporated into the brain sulphatide of 20-day-old rats when compared with adult rats. Maximum uptake of [^{35}S]sulphate coincided with the period of most active myelination in the brain

and it is therefore possible that at this time [^{35}S]-sulphatide is being incorporated into the developing myelin sheath. Labelled sulphatide persisted in the brain with little loss of radioactivity for at least 150 days after injection of neonatal rats. Similar metabolic stability has been demonstrated for cerebrosides and other typical lipids of the myelin sheath (Davison, Morgan, Wajda & Payling Wright, 1959). It is therefore suggested that sulphatide may be one of the most characteristic lipids of the myelin sheath. Relatively small amounts of [^{35}S]sulphate were incorporated into the lipids of the adult brain and this [^{35}S]sulphatide underwent slow turnover. This is consistent with the view that most of the adult brain sulphatide is metabolically stable and that [^{35}S]sulphate is only incorporated into functional cellular constituents in the adult brain. If the metabolically stable sulphatide is to be labelled, this can only be done during the course of development.

This hypothesis was discussed in relation to the work of Radin, Martin & Brown (1957) and Green & Robinson (1960) who studied brain sulphatide metabolism in adult rats.

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Radin, N. S., Martin, F. B. & Brown, J. R. (1957). *J. biol. Chem.* **224**, 499.

(9) Fatty Acids of Nervous Tissue

By R. W. R. Baker. (*Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.* 1)

Ester-linked long-chain fatty acids obtained by mild alkaline hydrolysis of lipids from human nervous tissue have been examined by gas chromatography. Results for total lipids (Baker, 1961) showed that the distribution of acids varied from one region of the nervous system to another. White and grey matter from brain differed chiefly in their contents of palmitic, oleic and longer-chain acids. The lipids from white matter yielded half as much palmitic but twice as much oleic acid as those from grey matter, and contained more of the C_{20} acids but less of the C_{22} acids. Spinal cord contained shorter-chain acids present only in traces in brain, while these were even more prominent in lipids obtained from sciatic nerve. The peripheral nerve lipids contained an appreciable quantity also of palmitoleic acid, but were almost devoid of acids of 20 or more carbon atoms, and in general resembled in acid composition the lipids of adipose tissue.

Fractionation of the total extractable lipids by chromatography on alumina columns has revealed differences within the fast-running, lecithin and kephalin fractions from different sites. Lecithin derived from white matter for example contained much more oleic but less stearic acid than that from grey matter, while the latter was richer in palmitic and arachidonic acids. The kephalin fraction from white matter was also much richer in oleic acid than the corresponding fraction from grey matter.

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(10) Determination of Magnesium by Atomic Absorption Spectroscopy

By F. W. Heaton and J. B. Dawson.* (*Medical Research Council Unit for Metabolic Disturbances in Surgery, The General Infirmary, Leeds, and Department of Medical Physics, University of Leeds*)

The development of flame photometry has greatly facilitated the estimation of sodium and potassium, but the application of this technique to the determination of magnesium is restricted by (*a*) relatively low sensitivity, and (*b*) interference produced by other constituents of biological materials.

The limitations of flame photometry are largely overcome by using the principle of atomic absorption spectroscopy suggested by Walsh (1955). An instrument suitable for the determination of magnesium by the atomic absorption method has been constructed in this laboratory. Light from a magnesium-discharge lamp is passed through an elongated acetylene–air flame; into the flame are aspirated solutions whose magnesium content is to be determined, and the light absorbed at the magnesium resonance line 285·213 mμ is compared with that absorbed by a standard magnesium solution treated similarly.

Magnesium may be determined in serum, urine and solutions of ash obtained from food or faecal materials after simple dilution; no deproteinization is required. Serum requires 50-fold dilution, dilution 100 times is usually satisfactory for urine. Standard and unknown solutions are acidified with hydrochloric acid to 0·1 N; the acid acts as a spectroscopic buffer and minimizes the effect of other components of the solution.

Optimum accuracy is obtained with solutions containing 0·02–0·08 mg. of magnesium/100 ml., the calibration curve being linear up to a magnesium concentration of 0·10 mg./100 ml. Depending on the number of specimens to be analysed, duplicate estimations require from 30 min. (1 sample) to 90 min. (4 samples), including the time required for

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preparation of samples and the calculation of results.

The method is virtually free from interference by inorganic ions. The organic constituents of samples cause slight enhancement of the apparent magnesium concentration; the magnitude of this effect varies from 0·5% with urine to 2·0% with plasma.

Walsh, A. (1955). *Spectrochim. Acta*, **7**, 108.

(11) A Bile Alcohol Sulphate from the Hagfish, *Eptatretus stoutii*

By G. A. D. Haslewood. (*Guy's Hospital Medical School, London, S.E.* 1)

Through the kindness of Professor D. L. Fox and Dr David Jensen, of the Scripps Institution of Oceanography, the bile of the Californian hagfish was made available. Seventy fish, collected for this work by Dr Jensen, gave 2·23 g. of crude bile solids. These readily yielded a crystalline 'bile salt', m.p. about 193° (decomp.), with a very bitter taste and behaving on paper chromatograms as having a main component much more 'polar' than taurocholate or the sulphates of ranol, cyprinol or 'scymnol'.

The 'bile salt', unlike others encountered previously, was highly resistant to alkaline hydrolysis, giving no SO_4^{2-} after 20 hr. at 114° in 2·5N-KOH. Heating at 100° in 0·25N-HCl caused some decomposition but gave sulphate ion and also, in poor yield, a crystalline neutral compound (*A*). Alkaline treatment, as above, but at 160°, gave in fair yield another neutral substance (*B*), together with SO_4^{2-} corresponding to an analysis for the 'bile salt' of S, approx. 8%. Substance *A* was more 'polar' than *B* on paper chromatograms: the infrared spectra (KBr disk) of *A* and *B* were similar but not identical, and neither showed evidence of a cholic acid nucleus. The 'bile salt' was easily purified as a crystalline barium salt, m.p. 148° (decomp.) which on paper chromatograms gave a single spot, corresponding to the main component of the original substance. The barium salt gave C, 42·1; H, 6·5% (analysis by Messrs Weiler and Strauss) and Ba (by precipitation as $BaSO_4$), 17·0%. The analytical figures, together with the origin and properties of the barium salt suggest the possibility of a disulphate formula such as $C_{27}H_{46}O_x(O{\cdot}OSO_3^-)_2Ba^{2+}, yH_2O$, where x is 3 or 4 and y is 1 or 2. The sulphate ester grouping may be $-\overset{25}{C}H{\cdot}(CH_2{\cdot}O{\cdot}SO_3^-)_2$: a similar group (as a monosulphate) is perhaps present in 'scymnol sulphate' (see Cross, 1960). The main bile salt is certainly an alcohol sulphate of an unusual type; it is especially interesting since the hagfish is described (Jensen, 1959) as 'among the most primitive of craniate chordates'. Bile of *Myxine glutinosa*, kindly provided by Finn Walvig, University of Oslo Biological Station, contained apparently the same salt, m.p. about 192° (decomp.).

Cross, A. D. (1960). *Proc. chem. Soc.* p. 344.
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(12) The Enzymic Liberation of Inorganic Sulphate from L-Serine *O*-Sulphate

By K. S. Dodgson, A. G. Lloyd and N. Tudball. (*Department of Biochemistry, University College, Newport Road, Cardiff*)

The hydroxylated amino acid, L-tyrosine, is known to occur in mammalian fibrinogens as the corresponding *O*-sulphate ester (see Bettelheim, 1954). Other hydroxylated amino acids have not so far been shown to occur in proteins as *O*-sulphate esters, although L-serine is present in some proteins in the form of the *O*-phosphate ester.

Attempts have been made to achieve the enzymic sulphation of L-serine, L-threonine and L-hydroxyproline by fortified rat-liver supernatant preparations which were able to sulphate other hydroxylated compounds. However, although a wide range of experimental conditions was used, no evidence of sulphation of the amino acids could be obtained. During the course of these investigations it became apparent that an enzyme system present in rat liver was able to liberate inorganic SO_4^{2-} from L-serine *O*-sulphate. The bulk of the enzyme activity was present in the high-speed supernatant fraction of liver homogenates which had been thrice frozen and thawed.

The activity of the enzyme was measured using L-serine *O*-[^{35}S]sulphate as substrate. After incubating the enzyme preparation with the labelled substrate in the presence of 0·25M-2-amino-2-hydroxymethyl-propane-1:3-diol–acetic acid buffer, a measured volume of the incubation mixture was subjected to paper electrophoresis in the presence of 0·1M-ammonium acetate. Suitable control determination, as well as markers of inorganic $^{35}SO_4^{2-}$ and L-serine *O*-[^{35}S]sulphate, were run on the same papers. After drying, the papers were cut into suitable strips and the radioactive zones were located and counted automatically with the aid of the Actigraph C 100 automatic strip-scanner. The areas of the inorganic $^{35}SO_4^{2-}$ and L-serine *O*-[^{35}S]-sulphate peaks on the scanning paper were then measured and used to calculate enzyme activity. Confirmation that enzymic liberation of inorganic $^{35}SO_4^{2-}$ was occurring was obtained in separate experiments in which the inorganic $^{35}SO_4^{2-}$ was precipitated and counted as $Ba^{35}SO_4$.

Maximum enzyme activity was obtained in the region of pH 7·0 when the concentration of sub-

strate was 0·01 M. Enzyme activity was slightly decreased in the presence of sodium acetate–acetic acid or phosphate buffers and was not significantly decreased by dialysis. The *O*-sulphates of L-threonine and L-hydroxyproline did not appear to be significantly affected by the enzyme. Preliminary experiments have shown that intraperitoneally injected L-serine *O*-[^{35}S]sulphate is appreciably desulphated by the rat.

It is not yet clear whether the responsible enzyme is a new sulphatase or whether an entirely different type of enzyme system, involving the L-serine moiety, is responsible.

This work was supported by a grant (A 1982) to K.S.D. from the Arthritis and Metabolic Diseases Division of the United States Public Health Services.

Bettelheim, R. F. (1954). *J. Amer. chem. Soc.* **76**, 2838.

(13) Molecular-Sieve Methods for Separations of Polypeptides according to Molecular Weight

By R. L. M. Synge and Mary A. Youngson. (*The Rowett Research Institute, Bucksburn, Aberdeen*)

Ionophoretic migration of polypeptides inside cellulose membranes impregnated with phenol–acetic acid–water (1:1:1, by wt.) was studied by Synge (1957), who observed a relationship of pore size with molecular weight. For working on a larger scale we used a multicompartment diaphragm cell in which the peptide cations have to traverse increasingly narrow-pored membranes, as in ordinary screening. The specimen compartment (3) was separated from compartment (2) by denitrated collodion similar to membrane CD (Synge, 1957); this from compartment (1, Pt cathode) by 'Ultracellafilter allerfeinst' (Membranfiltergesellschaft, Göttingen, Germany). The anode compartment (4) was separated from (3) by cellophan. The platinum anode was in a cellophan sac perfused with 0·1 N-H_2SO_4. Diameter of compartments and diaphragms was 3 cm. Compartments (length approx. 4 cm.) were all filled with above solvent mixture. Test material (5–10 mg.) introduced into (3) gave, after 6 hr. at 250 v, the following recoveries in (1) and (2) respectively: cytochrome *c*, 22%, 68%; insulin, 34%, 61%; gramicidin S, 39%, 48%; valine, 89%, 7%. Thus polypeptides having mol.wt. a few thousand may be selectively concentrated in (2).

Peptide concentrates such as these may be further fractionated by chromatography in the same solvent mixture on Sephadex G-75 (AB Pharmacia, Uppsala, Sweden. Cf. Gelotte, 1960; Porath, 1960). R_F values were: cytochrome *c*, 1·0; insulin, 0·92; gramicidin S, 0·58; valine, 0·45.

Theoretical reasons have been given (Synge, 1953, 1956) for the failure of polypeptides to dissociate from proteins and appear in extracts deproteinized by conventional procedures. In practice, hydrogen-bond-breaking solvents have proved necessary for isolating several polypeptide hormones. The phenol–acetic acid–water mixture is a powerful solvent for extracting proteins from tissues, and should simultaneously inactivate many enzymes. It is N-free, and can be effectively removed by repeated evaporation *in vacuo* with water. We hope that methods such as those described here will prove useful for systematically isolating polypeptides and related compounds from extracts of tissues and tissue components.

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(14) An Acetyl Peptide from Egg Albumin

By R. D. Marshall and A. Neuberger. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London, W.* 2)

The number of acetyl groups in whole egg albumin and in a glycopeptide separated from it was determined, and the values differed by one residue (Johansen, Marshall & Neuberger, 1960). A number of workers have shown that egg albumin has no free *N*-terminal amino group although it has a *C*-terminal proline residue. It has therefore been suggested that the molecule is cyclic or that its *N*-terminus is blocked by the carbohydrate moiety (Porter, 1950; Anfinsen & Redfield, 1956). It seemed possible from our findings that an acetyl group blocked the *N*-terminus of the peptide chain.

Heat-denatured egg albumin was digested with pepsin, followed by trypsin and chymotrypsin together. The digest, brought to pH 6, was evaporated to dryness, and extracted with absolute ethanol. The alcohol-soluble fraction, which was completely free of carbohydrate, was evaporated to dryness and chromatographed on Dowex-50 X4 H^+ resin (200–400 mesh). The material eluted by water was collected and shown to contain about 0·8 mole of tyrosine or a tyrosine peptide per mole of egg albumin by optical density measurements. The dinitrophenyl (DNP) technique indicated the absence of significant amounts of free amino groups. The material gave a negative reaction with ninhydrin. Electrophoresis on Whatman no. 4 paper at pH 2 (20 v/cm.) gave rise to a single immobile

band [Schwartz & Pallansch (1958) reagent], whilst at pH 6 most of the material moved as a single band towards the anode, leaving a faint mark at the origin. The nitrogen content was about 14·5 atoms per mole of 'tyrosine', the amino acids present after acid hydrolysis being aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, valine and leucine. No basic amino acids were identified. Chromatography (58 μmoles of 'tyrosine') on Dowex-2X8 resin (250–500 mesh) gave rise to fractions eluted by (i) water (20 μmoles), and (ii) 0·02N-HCl (25 μmoles), producing 1·15 and 1·30 moles of acetic acid per mole of 'tyrosine' respectively on acid hydrolysis. The HCl eluate contained about 8 atoms N per mole of 'tyrosine'. Aspartic and glutamic acids, serine, glycine, alanine, tyrosine, valine and leucine were identified.

Thus 0·77 acetyl residues have been isolated in peptide fractions that are completely free of carbohydrate. It seems reasonable to suggest that an acetyl group is the *N*-terminus of egg albumin.

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(15) The Effects of Papain on Cartilage and Chondromucoprotein

By G. Weissmann, J. L. Potter, R. T. McCluskey and L. Thomas (introduced by J. T. Dingle). (*Department of Medicine, New York University, New York, U.S.A.*)

The intravenous injection of 'inactive' crystalline papain protease (PSSP) causes widespread depletion of cartilage matrix in young rabbits. Paradoxically, the cysteine-'activated' enzyme (PSH) is ineffective *in vivo*, although both PSSP and PSH are effective *in vitro* (McCluskey & Thomas, 1958). Following injection of PSSP, a 50-fold increase of chondroitin sulphate in the circulation may be demonstrated by a turbidimetric technique using hexamminecobaltic chloride (Weissmann, Potter, McCluskey & Schubert, 1959). No such increase was demonstrated after injection of PSH. Using papain labelled with ^{131}I (which still reacted quantitatively with anti-papain serum) no difference could be shown in the rates at which the two forms of protease left the circulation. Depletion of cartilage matrix was caused by as little as 1–2 μg. of PSSP/g. of cartilage; less PSH entered the tissue. Zone electrophoresis on starch, and immunoelectrophoresis showed that both PSSP and PSH were bound by α_2-globulins of rabbit and human sera. Whether injected, or mixed *in vitro* with whole serum or isolated serum fractions, papain migrated with α-globulin, and not with albumin or γ-globulin. *In vitro* studies using purified bovine nasal septal chondromucoprotein (BCMP) demonstrated that serum prevented dissolution of this complex by papain. More complete binding of PSH by serum was held responsible for differences between the *in vivo* activities of the two preparations. As little as 1 μg. of PSH (with ethylenediaminetetra-acetic acid and cysteine) caused degradation of 100 mg. of BCMP in neutral aqueous solution. This effect was demonstrated by a fall in viscosity, changes in chromatographic and sedimentation properties, and the appearance of a protein-poor chondroitin sulphate fraction, as indicated by differential solubility in ethanol. BCMP does not migrate during paper electrophoresis or chromatography, whereas the papain-treated product migrates as chondroitin sulphate. Presumably a similar effect occurs in cartilage, where the large BCMP molecules (mol.wt. 4×10^6) are enmeshed by collagen fibres. Entry of a minute fraction of PSSP, but not of PSH, would lead to separation from protein of chondroitin sulphate, which can then readily diffuse into the circulation.

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(16) Identification of some Peptides from an Arginine-rich Histone, and their Bearing on the Structure of Deoxyribonucleoprotein

By D. M. P. Phillips and Pamela Simson. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.* 3)

The histones are probably attached by salt-linkages to deoxyribonucleic acid (DNA), and the phosphate groups, which are regularly spaced along the DNA chain, are probably neutralized by the basic amino acids. Various estimates indicate that 85–100% of the phosphate groups may be neutralized in this way (Davison & Butler, 1956; Vendrely, Knobloch-Mazen & Vendrely, 1960). The spacing (the number of residues) between the basic amino acids in histone is therefore of importance in determining the structure of deoxyribonucleohistone. The *average* spacing in most histone fractions is about three residues.

Preliminary experiments to establish the actual spacing have been carried out with tryptic digests of an arginine-rich histone from calf

thymus having 1 mole of *N*-terminal groups/18 000 wt., of which 0·95 mole was alanine (Johns, Phillips, Simson & Butler, 1960). A precipitate formed during the proteolysis, amounting to 25–30 % of the histone. It had a mol.wt. of 3000–5000, and only one basic acid for every seven other residues.

The supernatant was examined by paper electrophoresis and chromatography and the products so far identified (with the minimum yield as percentage by wt. of the histone) were: free lysine (1·6), free arginine (1·0), Ala.Arg (1·6), Ala.Lys (1·0), Leu.Arg (2·2), Gly.(Glu_2,Arg).Arg (2·4), and (Ser,Gly_2,Thr).Lys (2·2). The *N*-terminal groups of the supernatant peptides included four arginine and four lysine groups/14 000 wt. The *C*-terminal groups were arginine, lysine and a little alanine (alanine is *C*- and *N*-terminal in the intact histone), so that the known specificity of trypsin is operative, and basic acids occur in pairs in at least eight positions. Satake & Luck (1959) have already reported paired basic acids in *S. griseus* proteinase digests of another histone fraction.

These limited results cover about 35 % of this histone fraction and show that the spacing between basic amino acids can vary from 0 (juxtaposition) to at least seven residues. Parts of the deoxyribonucleohistone may therefore be like nucleoprotamine, and other parts may have loops of non-basic acids lying in a groove of the DNA double helix or, with the longer loops, forming bridges between separate double helices.

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(17) A Helical Polynucleotide Template Hypothesis for the Biogenesis of Rubber

By A. I. McMullen. (*The Natural Rubber Producers' Research Association, Welwyn Garden City, Herts.*)

*iso*Pentenyl pyrophosphate (3-methyl-Δ^3-butenyl 1-pyrophosphate) is now known to be incorporated rapidly and extensively into high-molecular-weight polyisoprene by fresh latex from *Hevea brasiliensis* (Archer, Ayrey, Cockbain & McSweeney 1960; cf. also Lynen, 1960). Dimerization of this 'active isoprene' is thought to proceed via a reactive cation intermediate formed by a prior allylic transformation (Lynen, Eggerer, Henning & Kessel, 1958; Agranoff, Eggerer, Henning & Lynen, 1959). Extension of this mechanism by a 'single-site' enzyme system to the synthesis of homologous high-molecular-weight, water-insoluble polymers seems unlikely for several reasons. Consideration is therefore given to a basically similar polymerization proceeding within a double helix polynucleotide as an 'assembly template'.

Assuming that 'active isoprene' first interacts with a simple nucleotide, losing the pyrophosphate moiety, then the possibility exists of the association of a large number of *iso*pentenyl nucleotides under the influence of high-molecular-weight 'template' nucleic acid. The formation of a double *iso*pentenyl polynucleotide helix could then ensue wherein the phosphate–sugar backbones are in an extended condition with a P–P spacing of about 7 Å and where the two chains are staggered relatively so that the separation of phosphate groups, alternating above and below the median of the narrow groove, is 3·5 Å with respect to this median.

A model has been constructed to illustrate that in this configuration the *iso*pentenyl units would be positioned in the groove in a highly favourable geometrical situation for $C_{(1)}$–$C_{(4)}$ interaction. The electronegative environment of the annulus would favour allylic transformation of these units under the influence of *iso*pentenyl isomerase (Agranoff *et al.* 1959) thus initiating a catalytic chain-like reaction which would result in simultaneous disruption of the *iso*pentenyl $C_{(1)}$–nucleotide bonds, interaction with the $C_{(4)}$ double bond of adjacent units and consequental *cis*-polymerization. The loss of bondage with the helix would require immediate removal of the hydrophobic rubber molecule from the template owing to the high interfacial energy involved. In the case of the *trans*-polyisoprene molecule, where the condensed linear repeat spacing is nearer 4·5 Å, it is envisaged that the specific template does not allow as close an approach of the subunits to the helix axis, possibly due to its having a narrower annulus.

This type of model might also be offered as an explanation of the particulate fraction requirement during biosynthesis of other insoluble $C_{(1)}$–$C_{(4)}$-linked linear high polymers such as chitin (Glaser & Brown, 1957) and cellulose (Glaser, 1957, 1958), and provides a simple and versatile method of obtaining a stable format of sufficiently large linear dimension to accommodate this kind of polymer, in an aqueous system.

The more complex kind of carrier polynucleotide which appears to be essential for coding activated amino acids during protein synthesis would not be necessary in these cases.

Preliminary evidence is available indicating a close association between rubber and 'insoluble' nucleic acid in fresh latex.

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(18) Configurational Changes of Muscle Proteins

By G. N. Graham. (*Department of Biomolecular Structure, The University, Leeds* 2)

It is generally accepted that the two muscle proteins actin and myosin and their complex actomyosin are in some way responsible for the events of muscular contraction. At the fundamental molecular level Astbury (1947) has suggested that contraction is a special manifestation of the configurational changes associated with the K-m-e-f group of proteins, namely the intramolecular folding of the polypeptide chains to produce the cross-β or supercontracted state. Pautard's (1959) recent demonstration that the cross-β configuration could be produced in actomyosin gels by treatment with adenosine triphosphate and changes in pH has lent support to this view. Hayashi, Rosenbluth, Satir & Vozick (1958) studied the contraction of artificially prepared threads of actomyosin containing various ratios of actin to myosin, and came to the conclusion that myosin alone does not contract with adenosine triphosphate and that actin is a specific factor for contraction.

In the light of these facts a study of the configurational changes brought about in actin by pH changes is indicated. Well-oriented X-ray diffraction photographs of purified actin are difficult to obtain consistently. Meanwhile, however, a start has been made using the intact myofibril. The wide angle diffraction pattern of glycerized rabbit psoas muscle (Szent-Gyorgyi, 1951) shows a clearly defined actin picture superimposed in the typical α-diagram contributed by myosin. The damp washed muscle fibres are treated with acetic acid vapour for 15 min. and allowed to dry at room temperature without tension. Following this acid treatment the typical actin meridional spacings disappear and the presence of a quite well defined cross-β picture can be clearly observed. Observations on the infrared spectrum of purified actin films give evidence of the presence of the β-configuration under conditions of low pH (Graham, Parker & Pautard, in preparation).

The effect of actin concentration on the dimensional changes of actomyosin gels has been studied using a measurement of floc volume on lowering the salt concentration, under standard conditions of low-speed centrifugation. An attempt is being made to correlate these changes with configurational changes as shown by X-ray diffraction and infrared spectroscopy. Preliminary findings of this work will be reported and the possible implications discussed.

It is a pleasure to thank the Muscular Dystrophy Association of America, Inc., for financial aid.

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(19) The Purification of Thyrotrophic Hormone

By M. L. Dedman, J. S. Fawcett and C. J. O. R. Morris. (*Department of Experimental Biochemistry, London Hospital Medical College, London, E.* 1)

The purification of thyrotrophic hormone from beef anterior pituitary glands has been studied using the thyroidal ^{131}I-uptake bioassay method of Querido, Kassenaar & Lameijer (1953) to follow the course of the fractionation. A hormone concentrate is prepared by saline extraction and isoelectric fractionation according to the method of Ciereszko (1945), with a specific potency of 0·6–0·8 i.u./mg. Further purification is achieved by chromatography on the cation-exchange resin Amberlite XE-97 at pH 7·2. Successive elution with 0·01 M- and 0·1 M-NaCl removes 75% of the inert protein, while the biological activity is eluted with 10–15% of the protein by 0·5 M-NaCl, yielding a product with 4–6 i.u./mg. This material is subjected to a further chromatographic purification on carboxymethylcellulose in formate buffer at pH 3·4. Of the total protein, 70% is eluted by 0·11 M-NaCl in 0·05 M-formate, while the biological activity is recovered almost quantitatively by elution with 0·2 M-NaCl in 0·5 M-formate, giving a material which after dialysis and lyophilization has a specific activity of 10–20 i.u./mg. This product is stable in the dry state, and has been purified further by several methods. In agreement with the experience of others (Bates & Condliffe, 1960; Carsten & Pierce, 1960) most methods appear to yield several fractions with biological activity. However, application of the new technique for the chromatography of proteins in urea solutions introduced by Wilson & Smith (1959) gives a three- to four-fold purification with essentially complete

recovery of activity in what appears to be a single major component. Chromatography is carried out on the cation-exchange resin Amberlite CG-50 at 4°, using 0·2 M-formic acid–M-urea at pH 2·0 as the eluent. The total biological activity is eluted with 20–35 % of the protein, while the remaining inert protein is firmly retained. The biologically active fraction is extremely labile, and can be lyophilized only in the presence of stabilizing agents. A typical product from starting material having 10 i.u./mg. had 30 i.u./mg. Reproducible behaviour is found on repeated chromatography. It is concluded that the apparent multiple forms of thyrotrophic hormone are complexes of a single active substance with inert components, and that these complexes are dissociated in urea solutions at low pH. The bearing of this phenomenon on the purification and properties of the hormone was discussed.

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(20) The Effect of Menadione (Vitamin K_3) on Tetrazolium-Reductase Activity of Rat Brain

By A. G. E. Pearse and R. Hess. (*Department of Pathology, Postgraduate Medical School, London, W.* 12)

Rat-brain mitochondria, isolated in 0·25 M-sucrose, were incubated in substrate media containing 3-(4:5-thiazolyl-2)-2:5-diphenyltetrazolium bromide (MTT). Addition of vitamin K_3 (20 μM) at pH 7·2 increased formazan formation by 83 % with α-glycerophosphate, 50 % with succinate, 60 % with diphosphopyridine nucleotide (DPNH) and 75 % with triphosphopyridine nucleotide (TPNH) as substrate. The reaction of both α-glycerophosphate and DPNH was enhanced further by freezing and thawing of the mitochondrial suspensions. Gradual loss of activity occurred on aging at 4°. Reduction of tetrazolium by the microsome-containing supernatant fraction was increased at rates comparable to those shown by the mitochondrial fraction using either DPNH or TPNH as substrate. Oxidation of DPNH at pH 6·24 with vitamin K_3 as final electron acceptor (Colpa-Boonstra & Slater, 1958) was linear for 15 min. in the presence of cyanide or azide. Under these conditions, formazan production was equimolar to DPNH oxidation in the absence of tetrazole.

All the activities leading to tetrazolium reduction were inhibited strongly by dicoumarol and, in this respect, they behaved similarly to purified brain diaphorase (Giuditta & Strecker, 1960) and liver diaphorase (Ernster, Ljunggren & Danielson, 1960). Dindevan (2-phenylindane-1:3-dione), which has been shown to exert antithyroid activity apart from counteracting vitamin K in prothrombin synthesis (Williams & Doniach, 1960), selectively inhibited the effect of K_3 on both DPNH– and TPNH–tetrazolium reductase. Amytal (an inhibitor of DPNH–ubiquinone reductase; Redfearn & Pumphrey, 1960) abolished only the effect of K_3 on mitochondrial formazan production. Results of histochemical enzyme reactions on fresh frozen tissue sections were consistent with the experiments performed *in vitro*.

In contrast to the view taken by others (Wattenberg & Leong, 1960), it is suggested that formazan formation via reduced vitamin K_3 indicates a pathway different from direct enzymic tetrazolium reduction and may involve flavoproteins with different properties.

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(21) Ubiquinone and Vitamin K_2 in Bacteria

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Freeze-dried cells from sixteen species of bacteria were extracted with *iso*octane and the lipids fractionated by chromatography on alumina and elution with suitable mixtures of ether and light petroleum. Vitamin K_2 and ubiquinone, where present, were sharply separated and estimated by measurement of absorption (in *cyclo*hexane) at 249 and 272 mμ respectively. Slightly lower values for ubiquinone were obtained by reduction with KBH_4 in ethanol and observing the fall in absorption at 275 mμ. Results are expressed below as μmoles of vitamin K_2 or ubiquinone per gram dry weight of organism. Respiratory activity was also determined as Q_{O_2} of washed suspensions using glucose or lactate.

Both substances were found in the Gram-negative *Escherichia coli* (K_2, 0·32; ubiquinone, 0·35) and *Proteus vulgaris* (K_2, 0·62; ubiquinone, 0·65). The other Gram-negative organisms contained no K_2 (below 0·05), but ubiquinone was present as follows: *Pseudomonas pyocyanea*, 1·40; *Neisseria catarrhalis*, 1·98; *Aerobacter aerogenes*, 0·53; *Pasteurella pseudotuberculosis*, 0·12; *Serratia marcescens*, 0·32; *Achromobacter hartlebii*, 0·43; *Azotobacter chroococcum*, 0·44. The strict anaerobe *Clostridium sporogenes*, and other two organisms showing no oxygen uptake (*Lactobacillus casei* and spores of *Bacillus subtilis*) lacked both K_2 and ubiquinone. The remaining Gram-positive organisms lacked ubiquinone (below 0·001) but vitamin K_2 was present as follows; *B. subtilis*, 0·7; *B. megaterium*, 0·66; *Staphylococcus albus*, 1·4; *Sarcina lutea*, 1·8; *Corynebacterium diphtheriae*, 0·75 (cf. Lester & Crane, 1959).

The oxygen-utilizing organisms thus contained either ubiquinone, vitamin K_2 or both; though no quantitative correlation was possible between Q_{O_2} and content of either quinone. Organisms without aerobic metabolism contained neither ubiquinone nor vitamin K_2; except that anaerobically grown *E. coli* and *Pr. vulgaris*, which showed no oxygen consumption, contained ubiquinone and vitamin K_2 at the same concentrations as the aerobically grown cells. The side-chains were determined by paper chromatography; ubiquinone was the C_{40} type (except for C_{45} in *Ps. pyocyanea*); vitamin K_2 was also C_{40} in *E. coli*, *Pr. vulgaris* and *Sarc. lutea*, but C_{35} elsewhere.

D.H.L.B. is in receipt of Agricultural Research Council Scholarship.

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DEMONSTRATION

Microchemical Identification of Certain Steroids

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Compounds of iodine with cholic acid (Barger & Field, 1912), cortisone (Zaffaroni, Burton & Keutmann, 1950) and certain other steroids (Bassil & Boscott, 1951; Heller, Hohensee & Hüttenrauch, 1958) have been recorded. Complexes of iodine–potassium iodide with other compounds containing a carbonyl group, such as benzophenone (Clover, 1904) and coumarin (Morgan & Micklethwait, 1906; Simonis, 1917) have also been described. Clarke & Taylor (1960) have demonstrated a test for progesterone based on a similar reaction. When an ethanolic solution of this compound is added to a 1% solution of iodine in 50% aqueous potassium iodide (Fulton, 1929), irregular rosettes of hair-like crystals are formed.

Further investigation has shown that this reagent may also be used to identify several other biologically active steroids. Thus, under the same conditions oestrone gives serrated plates, androsterone bundles of fine needles, *epi*androsterone rosettes of branching rods, which may later re-form into irregular conglomerations, and cholic acid, fans of fine blue needles.

The reaction is carried out by the hanging microdrop technique described by Clarke & Williams (1955). A cavity slide ringed with gum arabic solution is inverted over a coverslip on which a microdrop (0·1 μl.) of an ethanolic solution of the steroid has been mixed with a similar drop of the reagent. The slide is re-inverted and examined under low magnification. As the sensitivity of the test is of the order of 0·1–0·01 μg., it is particularly suitable for confirming the identity of a compound eluted from a paper chromatogram (Clarke & Hawkins, 1960).

In some cases (e.g. oestradiol-17β) crystals of the steroid itself are thrown out of solution by the addition of the reagent. These may be distinguished from crystals of an iodine derivative by repeating the test using water instead of the iodine solution, and comparing the crystals formed.

Little appears to be known about the structure of these compounds. Cramer (1951) suggests that they are clathrates, but Heller *et al.* (1958) postulate an oxo–iodine–hydroxy linkage.

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